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EDITOR-IN-CHIEF
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It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

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THE MANNITOL-PRODUCING ORGANISMS IN SILAGE

G. P. PLAISANCE AND B. W. HAMMER

From the Bacteriology and Dairy Sections of the Iowa Agricultural Experiment Station

Received for publication December 27, 1920

INTRODUCTION

The chemistry section of the Iowa agricultural experiment station has shown (Dox and Plaisance, 1917 a and b) that mannitol is a normal constituent of silage and has reported experiments indicating that "it is formed in silage fermentation by bacterial reduction of the fructose-half of the sucrose molecule." In silage, the mannitol is produced in considerable amounts, simultaneously with the acids, the carbon dioxide and the alcohol and "its presence accounts in large measure for the deficit noted when the sum of these products is balanced with the fermented sugar."

The results reported in the present paper[1] deal with the isolation from silage of organisms capable of producing mannitol when grown in pure cultures in corn, corn juice, and various other materials.

HISTORICAL

The presence of mannitol in the higher plants, in both the higher and lower fungi, and in various fermented materials such as wine, vinegar and sauerkraut, as well as its production by organisms, has already been dealt with in some little detail in the publications of the Iowa station. It is evident that mannitol fermentation has long been known and that it is more or less common.

[1] The work herein dealt with was carried out in 1917 and was to have been reported at the meeting of the American Society of Bacteriologists in that year. The National Research Council, however, requested that the report be delayed because of the possible use of mannitol in the manufacture of explosives.

METHODS USED

In order to prevent repetition, some of the materials used are here described. The corn juice was secured by pressing green corn, while the stover juice was obtained by soaking corn stover in water for twelve hours and then pressing. The corn silos were made by packing chopped green corn, and the stover silos by packing chopped stover, and adding a calculated amount of water and usually about 5 per cent sucrose, calculated on a dry basis. For most of the silos, the material was packed in quart Mason jars but in a few instances bottles or flasks holding from 1 to 2 liters were used.

Corn juice agar was made by adding 1.5 per cent agar and 1 per cent pepton to the corn juice while the stover juice agar was made by adding 1.5 per cent agar, 1 per cent pepton and 5 per cent sucrose to the stover juice; the stover juice agar was commonly cleared with an egg when it was wanted for plating but this was not necessary with the corn juice agar. The corn juice agar was more satisfactory than the stover juice agar because it was lighter in color and apparently gave a more satisfactory growth.

The method of isolating and determining mannitol in silage was that used in the former work at the Iowa station which has already been referred to. Mannitol was determined in liquid cultures by evaporating 100 cc. aliquot to dryness on a steam box; the residue was then extracted five times with boiling 95 per cent alcohol (about 15 cc. of alcohol in each portion) and the combined extracts filtered as soon as cold. After standing over night the mannitol had crystallized; the crystals were sucked dry, recrystallized from water and alcohol, dried and then weighed.

RESULTS SECURED

The rôle of microörganisms in mannitol production

Although in the previous work at the Iowa station the production of mannitol was secured by inoculating sterilized stover (plus sucrose and water) with a decoction of a leaf of corn silage

and was not secured in "antiseptic" silage made by adding ether to corn, it seemed desirable to repeat and extend these experiments in order to confirm the relationship of organisms to mannitol production. The inoculation of sterilized corn or stover silos with a bit of normal silage gave mannitol production regularly. The lack of mannitol in corn silos treated with various chemicals and held at room temperature for periods that gave mannitol with the untreated corn is shown in table 1.

When silage from the silos to which the various chemicals had been added was examined under the microscope a very few lightly stained bacteria were the only microörganisms observed and these, in all probability, represented organisms that were

TABLE 1

The influence of various chemicals on mannitol production, room temperature incubation

ADDED TO THE CORN	PERIOD OF HOLDING	MANNITOL
	days	
Nothing added, normal fermentation	14	Present
Ether	14	None
Chloroform	14	None
Chloroform and toluol	14	None
Formaldehyde	14	None

present on the corn at the time the silos were filled. The normal silage on the other hand showed very large numbers of well stained bacterial and yeast cells and thus presented a very different picture than the treated silage. When these statements are compared with table 1 it is evident that when microörganisms developed normally mannitol was produced, while when the growth of microörganisms was prevented by various chemicals no mannitol was formed.

The isolation of mannitol-producing organisms

The isolation of organisms capable of producing mannitol was attempted by plating out samples of silage on corn juice or stover juice agar. Representative organisms developing on the plates were grown on agar slopes (usually the same agar as

that used for plating) and were then tested for mannitol-producing power by inoculating into sterile corn juice, sterile stover juice, a sterile corn silo or a sterile stover silo, allowing growth to go on for a period varying from a few days to several weeks, and then examining the material for mannitol.

Attempts were made to isolate mannitol-producing organisms from a number of samples of silage that had been ensiled several months but only negative results were secured. Yeast[2] colonies were commonly present on the plates in considerable numbers and often made up the greater part of the developing flora; many of these were tried out for mannitol-producing power but when the yeasts were in pure culture mannitol was never found. Other types of silage were then studied and the first mannitol-producing organism isolated —M39— was secured from silage fourteen days old that was made by ensiling green corn from the greenhouse; the silage contained 1.09 per cent mannitol at the time it was plated out. The colony from which M39 was secured was very small and comparatively few of its kind were present; the organism was found capable of producing mannitol in sterile corn juice, in sterile stover juice, in sterile corn silos, and in sterile stover sucrose silos and has consistently given mannitol in a large number of trials.

Mannitol-producing organisms were readily isolated from a sample of corn juice that was covered with oil (to keep down mold growth) and allowed to ferment spontaneously and that showed, after a short period, considerable quantities of mannitol. A direct microscopic examination showed many yeast cells and still larger numbers of bacteria. On plating out, on corn juice agar, material from both the upper and lower layers of this fermenting juice, colonies similar to those of M39 were found in large numbers, together with many yeast colonies. When the colonies similar to those of M39 were streaked on agar slopes and then inoculated into either sterile corn juice or a sterile silo, mannitol was found in considerable amounts after the usual holding period.

[2] The term yeast is used to indicate organisms reproducing by budding.

Evidence that organisms of the type of M39 are concerned in the production of mannitol was furnished unexpectedly in two instances as follows:

1. A control stover silo, which had been opened after sterilization only for the purpose of adding sterile water, contained considerable mannitol when it was examined after a period of about two weeks. Microscopic examination showed many organisms morphologically resembling M39 and corn juice agar plates inoculated with the material yielded colonies like those of M39; transfers were made to corn juice agar and when the organisms were inoculated back into the usual test materials mannitol was found in considerable quantities after a holding period of from ten to twenty days. It seems that the presence of the mannitol-producing organisms in the silo was due to accidental inoculation since the organism is not exceptionally heat resistant and accordingly would not be expected to survive the heating during sterilization; moreover the other control showed neither organisms nor mannitol.

2. One of the yeasts isolated from silage was inoculated into a sterile stover silo and after the usual holding period an examination showed the presence of mannitol. When the silage was examined microscopically, in addition to the yeast, an organism morphologically similar to M39 was found in large numbers; this organism was isolated by plating on corn juice agar and proved capable of producing mannitol when inoculated into the usual test materials. Although a microscopic examination of the original yeast culture had shown no bacteria, it is entirely possible that a very small number of organisms of the M39 type may have been present in the culture and had thus been inoculated into the silo along with the yeast. Contamination of the silo, either at the time of inoculation or later when the silo was opened to release pressure, is another means of explaining the results secured.

Influence of oil at the surface of the liquid on mannitol production

Since the formation of mannitol is to be looked upon as a reducing process, it was thought that the addition of sterilized

oil to the flasks of liquids to be fermented might materially modify the results obtained. A number of comparisons of oiled and unoiled material were made at room temperature, using a juice expressed from cane and to which 2 per cent sucrose had been added before sterilization; the results secured are presented in table 2. From the data given it is evident that under the conditions employed, a larger percentage of mannitol was secured with oil than without it. Because of the gas liberated, which probably drives off much of the unconsumed oxygen, the conditions in the unoiled flasks must be, to a certain extent, anaerobic and this, in all probability, explains the formation of mannitol in the absence of oil. Whether the presence of air results in a smaller production of mannitol or in a destruction

TABLE 2

The influence of oil on mannitol production, room temperature incubation

ORGANISM	PERIOD OF INCUBATION	PER CENT OF MANNITOL	
		Without oil	With oil
	days		
M39	12	1.58	1.75
M363	12	0.60	0.83
M308	12	Trace	1.79
M439	12	0.44	1.60
M283	12	0.46	0.71

of a portion of that produced is impossible to determine from the data available.

In general, during the fermentation of the juices used there was a pronounced change in color; in the unoiled flasks the lighter portion involved mainly the lower depths, due presumably to the air above, while in the oiled flasks practically the entire liquid was involved. It seems probable that the change in color involved a reduction of some indicator present in the fermenting juice, the process being essentially similar to the reduction of litmus by many organisms, although it is possible that the change in color was due to the production of acid; the latter explanation is less acceptable than the former since the change in color in general agrees with the state of anaerobiosis.

Materials yielding mannitol

The organisms that were found capable of producing mann tol in the usual test preparations were studied as to their ability to produce it in a number of other materials. Two cultures from different samples of silage were inoculated into sterilized carrot juice[3] but no mannitol was secured. Both table and sugar beet juices were tried, two cultures on the former and four on the latter, but with negative results. Cabbage juice gave considerable quantities of mannitol with each of the three organisms inoculated into it and in general there was abundant gas formation. With the juices of the carrot, beet and cabbage there was a change in color similar to that secured with the corn and stover juices and in all cases the turbidity which developed indicated that the organisms were growing well. The failure to secure the production of appreciable amounts of mannitol with carrot and beet juice was undoubtedly due to the lack of the proper carbohydrate materials in them. A considerable number of flasks of apple juice were sterilized and inoculated with different cultures but there was no evidence of growth in any of them and the few flasks examined showed no mannitol; the same results were secured when the apple juice was neutralized before sterilization. Considerable quantities of mannitol were secured when sunflower stalks, leaves and blossoms were chopped, packed in jars, sterilized and inoculated with pure cultures of different mannitol-producing organisms, or when the sunflower material was packed in jars and allowed to ferment spontaneously. Cane yielded considerable quantities of mannitol when treated similarly to the sunflowers, as did also mixtures of stems, leaves and blossoms of dandelions.

One lot of stover juice (juice x) was found to contain only very small amounts of hexose sugars and accordingly it was used as a basis for testing out various materials. When 5 per cent cane sugar was added to this juice and the material sterilized, tests showed no appreciable inversion of the sugar; on inoculation with some of the mannitol-producing organisms

[3] The various juices were secured with a powerful press.

only traces of mannitol were found and it seems probable that this came from the small amounts of hexose sugars present in the juice. In the light of these tests it seems difficult to explain the value of the cane sugar in the stover juice, stover silos, etc.; it is entirely possible, however, that inversion might have occurred in some cases, even if not with juice x. By the addition of 5 per cent invert sugar to juice x, sterilization, and the inoculation of mannitol-producing organisms considerable quantities of mannitol were secured; growth was apparently very rapid and there was usually a pronounced change in color and the evolution of considerable gas. When honey (usually 8 per cent was used) was added to juice x, there was

TABLE 3

Mannitol in silage made from stover plus various substances, room temperature incubation

MATERIAL ADDED TO STOVER	MANNITOL
	per cent
Glycerol	0
Galactose	0
Glucose	0
Fructose	3.71
Maltose	0
Lactose	0
Inulin	0.40
Starch	0

an exceptionally heavy gas production, a pronounced change in color and the formation of considerable quantities of mannitol.

A series of silos was made by cutting up corn stover (containing practically no sugars) adding various substances, packing in Mason jars and then sterilizing, after which organism M39 was inoculated. The results of mannitol determinations made on the silage after a suitable holding period at room temperature are shown in table 3. From the data presented it is evident that fructose and inulin yielded mannitol while glycerol, galactose, glucose, maltose, lactose and starch did not. Many lots of silage made by inoculating various organisms into sterilized stover plus sucrose have, as already stated, yielded mannitol.

It seems then that only fructose, or materials giving fructose on hydrolysis were capable of yielding mannitol when acted on by the mannitol-producing organisms studied. The small amount of mannitol produced in the inulin stover silo, as well as in many of the sucrose stover silos, was undoubtedly due to the inability of the organisms to hydrolyze these materials and to the small amount of hydrolysis which occurred during the process of sterilization. The variations in the amounts of mannitol produced in the sucrose stover silos were very likely due to differences in the amount of hydrolysis, and this was materially influenced by the amount of acid present and by the extent of the heating. Gayon and Dubourg (1894; 1901) found that only fructose or its moiety yielded mannitol and Brown has shown how the configuration of fructose is such that it alone can be changed to mannitol by organisms.

It is entirely possible that certain organisms may be able to produce mannitol from such materials as sucrose and some results have been secured which indicate that one of the cultures isolated is able to do this. It seems quite certain, however, from results secured with the use of bouillon to which sucrose was added, that most of the cultures isolated are unable to change sucrose to mannitol.

Distribution of the mannitol-producing organisms

Since mannitol is a normal silage constituent and is produced by the action of microörganisms, it would be expected that mannitol-producing organisms would be rather widely distributed about barns where silage is used, due to the scattering of silage and of manure from animals consuming silage. A number of materials have been tested for mannitol-producing organisms by inoculating them into sterile corn juice, flooding the juice with sterile oil in order to keep down the growth of molds and then determining the presence or absence of mannitol after a suitable incubation period at room temperature. The production of mannitol has been secured with soil from a farm yard and also with milk, but the trials made are too few in number to allow of any conclusions regarding the extent of the contamination of these materials.

The per cent of mannitol produced in various materials

The per cent of mannitol produced in different materials varied widely. While this was due to a large extent to variations in the per cent of total sugar, as well as to variations in the make-up of the sugar in the original materials, differences in the efficiency of the different organisms tried undoubtedly played a very large part. Table 4 presents data, other than those already presented, showing the per cent of mannitol produced under different conditions and in various materials. Many other determinations were made only to find out whether mannitol was present in traces or in considerable quantities and the results are of course omitted.

TABLE 4

The per cent of mannitol produced under different conditions, room temperature incubation

MATERIAL	INOCULATION	PERIOD OF HOLDING	MANNITOL
		days	*per cent*
Cane juice plus 2 per cent sucrose	M283 plus yeast	12	1.85
Cane juice plus 2 per cent sucrose	M283 plus yeast	12	1.05
Cane juice plus 2 per cent sucrose	M308 plus yeast	12	1.65
Cane juice plus 2 per cent sucrose	M308 plus yeast	12	1.00
Cane juice plus 2 per cent sucrose	M439	12	0.49
Cane juice plus 2 per cent sucrose	M308	12	0.52
Green corn silage	M39	18	0.59
Corn juice	M393	12	0.60
Corn juice	M393	20	0.92

The destruction of mannitol

The data already reported by the Iowa station show that, in a silo, part of the mannitol produced is destroyed. Table 5 shows the per cent of mannitol present at various times in stover silage containing sucrose inoculated with organism M39 and held at room temperature. A series of silos were prepared and a different one used for each determination.

From table 5 it seems that, at least with the organism used, the production of mannitol was accompanied or followed by its partial destruction.

Organisms having mannitol-producing powers

A considerable number of organisms capable of producing mannitol were isolated from various samples of silage and studied morphologically, culturally, and biochemically. The results showed that the organisms cannot be considered to be of one type. Most of the cultures isolated produced no appreciable change in milk and undoubtedly should be classed as *Bacillus manniticus* of Gayon and Dubourg, but one of the cultures in particular produced a coagulation in litmus milk with an extensive reduction of the litmus and its general characteristics indicated that it should be classed as *Bact. casei*. Certain of the rod-shaped (Kruse 1910; Orla-Jensen 1919) lactic acid

TABLE 5

The per cent of mannitol at various times in stover silage containing sucrose and inoculated with organism M39, room temperature incubation

PERIOD OF HOLDING	MANNITOL
days	*per cent*
6	1.41
8	2.41
10	2.63
12	1.60
14	1.54
20	1.38

organisms have been shown by a number of investigators to produce mannitol. With this group of organisms, however, mannitol production is not a general characteristic since a considerable number of cultures from sources such as milk, silage and cow feces were examined for mannitol production by inoculating into satisfactory media but only with negative results.

From the findings reported it seems that the production of mannitol in silage is not the result of the action of organisms present in silage alone but is brought about by the activity of organisms that have been shown to produce mannitol in other materials such as wines, etc. The conditions, such as a lack of oxygen and the presence of sugar, existing in silage during the period of active fermentation are undoubtedly very favorable

to the type of change resulting in the formation of mannitol from fructose. The rod-shaped lactic acid organisms constitute a group that is present in silage in enormous numbers (Hunter and Bushnell, 1916; Sherman, 1916) and while many of these do not produce mannitol it seems probable that mannitol-producing forms may be expected among them.

CONCLUSIONS

1. The production of mannitol in silage is very evidently due to bacterial action.

2. Mannitol-producing organisms were readily isolated from silage, provided it had been ensiled recently. They were also secured from a sample of fermenting corn juice.

3. In liquids, more mannitol was produced when they were flooded with oil than when they were not.

4. Mannitol was produced, by the organisms isolated, in the juice of cabbage and in silage made from corn, sunflowers, cane or dandelions, but not in the juice of carrots, beets, or apples. Fructose, or materials giving fructose on hydrolysis, such as sucrose or inulin, also yielded mannitol when added to stover before sterilization; it is probable that the hydrolysis was due to the heating and the acid present and cannot be accomplished by the organisms although there may be variations among the organisms in this respect. Glycerol, galactose, glucose, maltose, lactose and starch did not yield mannitol when added to stover before sterilization while honey gave large amounts.

5. The mannitol-producing organisms were found to be present in soil from a farm yard and in milk.

6. The per cent of mannitol produced in different materials varied widely, due undoubtedly to a large extent to variations in the types and amounts of sugar present.

7. With the only organism that was tried, the production of mannitol was accompanied or followed by its partial destruction.

8. The organisms producing the mannitol in silage cannot be considered to be of one type.

REFERENCES

Dox, Arthur W., and Plaisance, G. P. 1917a The occurrence and significance of mannitol in silage. Jour. Am. Chem. Soc., **39**, 2078.

Dox, Arthur W., and Plaisance, G. P. 1917b The occurrence and significance of mannitol in silage. Ia. Agr. Exper. Sta. Res. Bul. 42.

Gayon, U., and Dubourg, E. 1894 and 1901 Sur les Vins Mannités. Ann. Inst. Pasteur., **8**, 108; and Nouvelles Recherches sur le Ferment Mannitique. Ann. Inst. Pasteur., **15**, 527.

Hunter, O. W., and Bushnell, L. D. 1916 The importance of *Bacterium bulgaricus* group in ensilage. Science, n.s., **43**, 318.

Kruse, W. 1910 Allgemeine Mikrobiologie, p. 401.

Orla-Jensen, S. 1919 The lactic acid bacteria. Mémoires de l'Académie Royale des Sciences et des Lettres de Danemark. Section des Sciences, 8^{me} série, **5**, no. 2.

Sherman, James M. 1916 A contribution to the bacteriology of silage. Jour. Bact., **1**, 445.

PRINCIPLES CONCERNING THE ISOLATION OF ANAEROBES

STUDIES IN PATHOGENIC ANAEROBES. II

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The subject of the isolation of anaerobes is one which the worker is inclined to approach with apologies. Every month or so a paper appears in some journal in which a new and expeditious procedure for the separation of anaerobes is described. There are many successful ways of isolating anaerobes and it is unwise to recommend any one method above all others. I have succeeded with various arrangements; and wish in this paper to analyze some of the principles governing the isolation of these organisms and to explain a few of the pitfalls which have caused many workers to believe that the securing of "absolutely pure" anaerobic cultures is a difficult matter. With a little practice and with the exercise of much discrimination, anaerobes may be isolated as quickly, or nearly as quickly, as aerobes.

Contamination occurs somewhat more frequently in anaerobic cultures than in those of aerobes. Contamination of originally pure cultures may be attributed to the following causes: (1) Insufficiently sterilized media; anaerobe media are usually pasty and require more careful sterilization than others. (2) Inoculation transfer involving the exposure of the cotton plug and of the inoculum to the air. I have noted in working in London and near the sea in San Francisco, that the more dusty the air, the more frequent are contaminations, and the contamination flora may vary according to location. (3) During incubation in closed jars the cotton plugs may become sufficiently moist for

molds to grow through them; where a mold can grow a bacillus can follow. (4) During prolonged incubation water of condensation may even run into the tubes from the top of the jar. (5) If stored in closed cans molds may grow through the plugs. Workers should take these points into consideration in planning their work. Anaerobic jars are exceedingly convenient and practical for periods of incubation under four or five days, and for much anaerobic study twenty-four to forty-eight hours incubation is sufficient. Prolonged incubation should be made under vaseline or in the case of sugar-free media in exhausted sealed tubes. Sealing of tubes is inadvisable where carbon-dioxide may be so confined that it produces an acid end-point. Re-incubation of cultures in exhaust jars should be cautiously undertaken so that the medium may not boil up to the cotton plugs. Anaerobic jars which do not require exhaustion are preferable for re-incubation of cultures.

The commonest contaminators of my cultures have been cocci and molds, not anaerobes. The reason that anaerobic contamination of anaerobe cultures is so very common probably lies principally in the uncritical handling of such cultures. If a coccus or mold contaminates a culture the worker immediately kills such an organism, but if an anaerobe enters the tube it proceeds to multiply unmolested. Daily watchful observation of the cultures studied is absolutely necessary for successful anaerobic work. I have not found indications of any so-called symbiotic tendency that makes anaerobes more difficult to isolate than aerobes.

Anaerobes vary greatly in their behavior and requirements, and the method of isolation must be adapted to the problem in hand as it turns up. Each combination of two or more species of organisms presents different elements for consideration and for adaptation of technique. There is no one method that is always best, and it is only after a worker knows something about the nature of the particular organisms that he is dealing with, their cultural behavior, and their morphology in the medium in which he regularly grows them, that he is able quickly and surely to isolate numbers of strains.

It is, of course, desirable to make use of methods that may be applied to the largest possible number of species, that are easy of manipulation, and moderate as to cost of time and material.

The organisms present in material to be investigated may belong in any one of four large groups, which may be described as follows:

ORGANISMS	UNDESIRABLE	DESIRABLE
Easily killed by heat	Non-sporulating aerobes, common, many species	Non-sporulating anaerobes: Welch bacillus is, in most media, the chief consideration
Not easily killed by heat	Sporulating aerobes, not common in pathological material; species numerous, however	Sporulating anaerobes, species legion in number

Whatever be the material that is to be investigated, a microscopic examination of a Gram stain is first in order. Practice only will enable the worker to form judgments which will be of value to him. As hints to the beginner, one may suggest that there are an endless number of species of anaerobes and that specific diagnosis by microscopic examination is futile. There are frequently many species of anaerobes in the material that finds its way to a laboratory, and, unless a study of many strains is intended, the isolation or demonstration of a single species, whose nature is guessed at, must be attempted. If the microscope shows the probability of the presence of that species, matters are simplified. To seek a certain organism one should familiarize himself with a pure strain of that type of organism, or study photographs or drawings of it; verbal descriptions are not of much value. He should also learn the colony form of *several strains* of the type he desires to obtain. The employment of a medium in which the morphology of the organisms is varied and characteristic is imperative. This laboratory uses chopped meat medium containing 5 per cent peptic digest broth (pH 7.2) for routine cultivation and this medium excels all other autoclaved media in the above respect. The use of oil over the medium to

produce anaerobiosis should be avoided whenever possible for routine work, as it interferes with the making of satisfactory smears; for long incubation and under certain circumstances demanded by technical considerations, vaseline will be found very useful. Ghon and Sachs recommend the use of agar for stratification; liquid media should be frozen before the agar is poured.

Heating. To free sporulating organisms from non-sporulating organisms heating is always resorted to. Heating of inoculum may be performed in one of two ways. Heavily inoculated media may be heated to 80° in a water bath for fifteen to thirty minutes. This method is highly inaccurate, especially in case pasty media are used, but it serves on occasion. Or the material to be inoculated may be heated in a Pasteur pipette after the following fashion:

Sera, exudates, and muscle extracts should be diluted with sterile saline. Cut the end of a Pasteur pipette off square with a file, flame it, then draw up the inoculum for about two inches by capillary attraction, and seal the pipette with less than a quarter of an inch of air space between the tip and the liquid. To kill non-sporulating organisms heat in a waterbath for ten minutes at 70° to 72°. Then flame the pipette above the inoculum to kill organisms that may have been above the water-line, mark the tip in several places with the file or diamond, slowly flame the tip, insert it in the tube of fresh medium, flame a pair of light forceps and with them break the tip of the pipette against the inner wall of the tube and expel the material.

If a worker is certain that the type of sporulating anaerobe desired is always highly resistant to heat, he may use higher temperatures, in the neighborhood of 100°C. for heating his cultures. Dr. K. F. Meyer informs me that he has repeatedly employed this method with success in the isolation of *Bacillus botulinus*. Von Hibler sowed mixtures containing such organisms, and even less resistant ones, directly into hot agar. Some strains of *B. botulinus* and of Novy's bacillus are highly resistant to heat.

1. To segarate non-sporulating anaerobes from aerobes

1. Heat to 56°-58° for five or ten minutes. This occasionally serves the purpose.

2. Try to induce sporulation by growing the mixture on alkaline sugar-free medium, such as alkaline egg, or serum medium (von Hibler, 1908, p. 189). When the anaerobes form spores, heat. This procedure is a sure method of freeing *B. Welchii* from ordinary aerobes: incubate for four days. This organism is found in a sporulating condition in soil and in fecal material.

3. Try a pathogenicity test. If the organism sought is pathogenic it may be recovered in pure culture from the animal tissues. Use this method for *B. Welchii, B. egens, B. fallax.*

4. Use selective media. For the Welch bacillus use milk or 1 per cent glucose broth. Inoculate it with a pipette, a fresh tube of medium every twelve hours if possible.

5. Use good anaerobic methods. Cultivate the material on meat medium in strict anaerobiosis, inoculate in agar dilution tubes that have been thoroughly boiled, and fish the colonies. This technique is described on page 461.

6. Northrup suggests the use of a 25-cc. burette, in which the organisms of an inoculated mixture will, on short incubation, sort themselves out, the aerobes growing above, the anaerobes below, where they may be drawn off through a stopcock.

II. To separate non-sporulating anaerobes or reluctantly sporulating anaerobes from other sporulating anaerobes

1. Use selective media, milk, with short incubation periods, for *B. Welchii.*

2. Use animal inoculation.

3. Use shake cultures.

4. Use semi-anaerobiosis: The non-sporulating anaerobes are naturally more resistant to oxygen than the sporulating ones.

Aside from *B. Welchii* this sort of organism is rarely sought after or noticed. Few non-sporulating anaerobes are described, and the group has been generally neglected, but careful methods show that non-sporulating anaerobic rods and cocci are not uncommon.

III. To separate either variety of anaerobes from sporulating aerobes

Sporulating aerobes are rather infrequently found in pathological material. One meets them frequently, however, in a medium that has been insufficiently sterilized. My encounters with sporulating aerobes have been so rare that it would be wise to recommend that a worker always go back to the original material and test it for the presence of any sporulating aerobe that he finds in a culture with which he is working. Avoid sporulating aerobes, do not contaminate cultures with them, and isolate the anaerobes from the original material again.

1. Sporulating aerobes are of two classes: strict aerobes (any good anaerobic technique followed by a colony method will free a culture of these) and facultative anaerobes. I have never encountered a sporulating facultatively-anaerobic aerobe that grew better under strictly anaerobic conditions than its accompanying anaerobes. Any strictly anaerobic colony method that will separate anaerobes from each other will separate them from aerobes. In my experience trouble with abundantly growing aerobic organisms denotes faulty anaerobiosis: the presence of a small amount of oxygen that permits the undue multiplication of the aerobes. My experience has, however, been almost entirely with pathological material and I may have failed to meet with the most troublesome aerobic organisms.

2. Kitasato and Weyl found that anaerobes were less sensitive to pyrocatechin, chinon, sodium formate, and sodium sulphindigotate than were the aerobes causative of cholera, typhoid and anthrax. Rivas continued this type of investigation.

3. Churchman has investigated the inhibitive effect of gentian violet on aerobic growth. Hall recommends the use of gentian violet in a dilution of 1:100,000 to separate sporulating aerobes from anaerobes. This, I should think, would work very well for the heavy Gram positive organisms of the *B. subtilis* group, provided the desired anaerobe is not of the same nature.

4. The spores of aerobes may sometimes be satisfactorily germinated in broth in a Petri dish, the broth being then heated and inoculated into agar.

IV. To separate sporulating anaerobes from non-sporulating anaerobes and aerobes

Heat as described on page 448.

V. To separate sporulating anaerobes from other sporulating anaerobes by cultural methods

I. *Heating.* The following diagram shows how heating may be employed:

ANAEROBIC BACTERIA	PROTEOLYTIC GROUP	NON-PROTEOLYTIC GROUP
Early sporulating species (18–24 hours)	Bifermentans group *et alii.* Do not occur very frequently	Nearly all sporulating organisms
Later sporulating species (24–48 hours)	Sporogenes group *et alii*	
Late sporulating species (48 hours on)	Tetanus group, botulinus group, *et alii*	

This diagram shows that if proteolytic early-sporulating organisms are absent, as is frequently the case, a saccharolytic form may be isolated or be rendered relatively far more abundant by heating eighteen to twenty-four-hour cultures successively. I have had mixtures of *B. sporogenes* and organisms of the blackleg group that were not pathogenic for guinea-pigs because of the scarcity of *B. Chauvoei.* Two successive heatings and inoculations made blackleg the predominant organism and the culture was highly pathogenic. This method is also excellent for organisms of the vibrion-septique group and for many non-pathogenic saccharolytic bacteria, as well as the early-sporulating proteolytic ones.

II. *Selective media.* Isolation methods usually depend on securing a predominance of the organism sought. To increase the relative numbers of an organism with whose nature one is familiar, a medium should be selected on which the organism grows best. For saccharolytic species mixed with proteolytic ones, use sugar-containing media. Meat medium plus 1 per cent glucose is

good, meat medium not neutralized in the making is also good. Ordinary meat medium, the culture being taken early in its development, is usually sufficiently selective. Sugar media selective for certain groups may be used, if the number of cultures to be isolated warrants the investigation of the sugars split by that group. I have found that culture in casein-digest liver-broth renders blackleg the most able guinea-pig invader in a blackleg-vibrion-septique mixture. To increase the percentage of proteolytic organisms use meat medium or brain medium in a culture two to four days old, or even older; or employ the medium of Achalme-Passini, salt solution or broth containing cubes of egg-white; or use serum medium or other sugar-free media; or a medium made up at pH 8.0 or above. For an organism whose morphology interests one and whose nature is not known, experiments should be tried with various media, and the behavior of the mixture should be studied. Under laboratory conditions certain types always tend to disappear from mixed cultures. It must be kept in mind that conditions must exist in nature which favor the multiplication of such species or they would have died out long ago. For such organisms try media of vegetable origin.

In taking samples of pathological material enrichment with the tissue in which the organisms are found is advisable. Schottmüller isolated septicemic streptococci in blood-glucose agar shakes. The many tissue-containing media favor the growth of pathogens. (Media summarized by Pfuhl.) Tunnicliffe used serum and ascites agar for the anaerobic coccus found by her in measles cases. Plotz and his co-workers added ascites or hydrocele fluid to glucose agar for blood cultures from their typhus patients. Dick and Henry employed blood-glucose agar for the various anaerobes found in the blood of scarlet fever patients. Leucowicz used serum-sugar agar for *Fusiformis*.

Digest media are excellent for anaerobes. A number of such media are discussed by Stickel and Meyer.

Serious problems sometimes arise. Thus, *B. tetani* is particularly difficult to isolate from gross mixtures, as it is not a tissue invader, and because it sporulates later than the organisms that usually accompany it. In case an organism like *B. tetani* grows

excellently on a given medium but its accompanying organisms grow better than it does, try similar selective media of modified reaction, or make use of exhaust media of the type recommended by Tulloch. One may always grow the objectionable species or several species in a medium till growth ceases, filter the medium and then grow the mixed culture in the filtrate. In case this fails one may add a minute quantity of some solid protein for a starter. Tulloch added a bit of rabbit kidney to an exhaust filtrate and found it highly selective for *B. tetani*. Von Hibler grew mixtures containing *B. tetani* on clotted rabbits' blood and stated it to be selective for that organism. But his photographs of the organism show his cultures to have been so badly contaminated that he may have been mistaken.

I have found a modification of a medium of Beijerinck's excellent for the enrichment of soil anaerobes (sodium phosphate 0.05 per cent, ammonium sulphate 0.05 per cent, soluble starch 1 per per cent, calcium carbonate 0.5 per cent). The anaerobic flora obtained in such a medium after heating a soil emulsion is very different from that obtained in meat or other media of complex composition. By fishing large lenticular or modified lenticular colonies from 2 per cent agar shakes of this medium which have been incubated for four days, the large butyric acid bacteria of the genus *Clostridium* may be isolated with comparative ease. Winogradsky (1902) recommends the use of media free of fixed nitrogen for the isolation of nitrogen fixing anaerobes (*Clostridium Pastorianum*); this medium is described by Fred (1916) and Bredemann used it for the isolation of his *Bacillus amylobacter* which he considers to be the same organism as Winogradsky's. Milk may also be used as an enrichment medium for many organisms of this genus.

Omeliansky (1904) describes the following method for enriching cellulose fermenters: Place in a long-necked flask any cellulose substance, paper, cotton, flax; add chalk, and fill to the top with water which contains 0.1 per cent ammonium phosphate, 0.1 per cent calcium phosphate, 0.05 per cent magnesium sulphate, and a little sodium chloride. Inoculate with slime or horse manure, cover, and set in the dark. In other publications (1895;

1902) he gives other formulae; several are given by Fred. Ankerschmitt used physiological salt solution containing cubes of potato to enrich splitters of hemicellulose. Choukevitch employed 1 per cent pepton broth with 5 per cent starch for starch splitting organisms. Silicate jelly as a substrate for such of these organisms as will not grow on agar is described by Omeliansky (1899) and formulae for similar jellies are given by Fred and by Küster.

III. *Symbionts* have been used to enrich certain types of anaerobes. Sturges and Rettger found that *B. putrificus* flourished best in the presence of *Bact. coli*, and used the latter as a symbiont for the former. Rhein used *Bact. faecalis-alcaligenes* as a symbiont for anaerobes, cultivating them in the presence of air: this organism has several advantages. Wilson and Stere describe a cocco-bacillus which is an excellent anaerobe symbiont.

IV. Another resource is to test the *resistance* of the desired species to *unfavorable circumstances*. Thus McCoy and Bengtson of United States Public Health Laboratory isolated many strains of tetanus with great ease by heating toxic strains at 70° for a half hour and inoculating the spores in veal agar dilution shakes. This technique is adverse for an anaerobe, but *B. tetani* appears to be hardy enough to withstand it. Modified highly acid or alkaline media, or media poor in protein may be used for such purposes. A pure strain of the desired organism is invaluable in testing out media of this sort.

V. *Aniline dyes* may be used to eliminate certain species of organisms and the possibilities which they offer are almost unlimited.

VI. *Selective temperatures* may be employed for enrichment of various organisms. *B. botulinus* was long thought to produce toxin at low temperatures only, because the contaminating organisms in the cultures outgrew it at 37°. Thermophilic organisms are of various types, and are discussed by Bergey. Major W. J. Tulloch tells me that the flora obtained by incubating a mixture of anaerobes in meat medium at 42° is quite different from that obtained at 37°, slender, oval end-sporing organisms predominating. It is probable that anaerobic organisms will be found that grow at much higher temperatures than at 42°.

VII. *Separation of organisms before sowing* was suggested by Stoddard, who shook his material with sea sand to separate encapsulated or autoagglutinated organisms. Dr. K. F. Meyer tells me that he has found such technique useful in isolating anaerobes from soil and from old meat cultures which had sporulated heavily. Such separation is not necessary when fresh cultures are used.

VIII. *Isolation by various colony methods.* Because of the confusion that exists as to the purity of cultures of anaerobes, it will be well to study the biological factors involved in the genesis of bacterial colonies. A colony is an aggregation of organisms that are prevented from mixing with other organisms by a physical obstruction. A colony may be defined as follows:

a. From one single organism—the ideal colony for isolation purposes.

b. From two or more organisms descended directly or indirectly from one organism—a satisfactory colony for isolation purposes.

c. From two or more organisms of closely related strains—the most undesirable type of colony for isolation purposes.

d. Or from two or more organisms of unrelated strains—an undesirable type of colony for isolation purposes. This type or a contaminated pure colony is sometimes useful in procuring a new proportional mixture of strains.

Broadly speaking a colony may consist of any number of organisms from one to infinity. Technically speaking a colony consists of the organisms confined within a certain radius inside of or on the surface of the mass of colloid gel. For purposes of discussion, let us define a bacterial colony as the uncontaminated descendants of a very small number of organisms, irrespective of the medium in which they are found. It will be realized that this definition covers perfectly the biological factors involved in the derivation of any ordinary agar or gelatin colony.

Colony methods available for the isolation of anaerobes are the following:

I. Agar colonies—von Hibler and older workers used also gelatin.
   A. Surface colonies.
   1. On plates.
   2. On tube slants.
   B. Deep colonies.
   1. In Petri dishes.
   2. In deep agar tubes.
II. Colonies in liquid media.
   A. Isolation of a single bacillus by the India-ink method.
   B. Isolation of a single bacillus or of a small number of organisms by the technique of Barber.
   C. Isolation of a single bacillus by the technique of Schouten, of Hecker, of Holker, or that of Malone.

Isolation from surface colonies has been employed by many workers with anaerobes. Veillon and Zuber list a large number of types of anaerobic apparatus, von Hibler (1908) gives a bibliography of various plates and apparatus for purposes of anaerobic culture, Besson's textbook figures a number of arrangements, and Fildes describes various methods at the end of McIntosh's report.

Henry uses plates of agar which he streaks with egg-albumen and incubates in hydrogen. Stoddard uses slants made of the modified egg medium of Stitt, made with tryptic broth and 1 per cent glucose. Zeissler, who at first used glucose agar plates containing human blood for the isolation of anaerobes, later employed horse blood and sheep blood agar plates. Many laboratories now make use of large slants of blood agar, kept under anaerobic conditions for the isolation of anaerobes. Isolation of nitrogen fixing organisms was accomplished by Winogradsky by inoculating cultures on pieces of carrot which he placed in vacuo, and Fribes isolated pectin fermenters on potato slants rubbed with chalk. McIntosh prefers agar slants to plates for isolation procedure. He reiterates: "It cannot here be impressed too strongly on the worker that the purity of a culture can only be tested and controlled by repeated surface cultivation," and he speaks of the Veillon-tube method of continental workers as giving impure cultures. I have used plating occasionally and am familiar with technique necessary to make anaerobes grow on plates. In fact

it was the first method for the isolation of anaerobes that I learned to use. It is a perfectly feasible method, but I find it to be less satisfactory than others for various reasons.

The difficulty of regulating the amount of moisture on the surface of the plate or slant is the primary drawback to the use of surface methods. Aerobic cultures differ fundamentally from anaerobic ones in this respect. They are, so to speak, self-regulating in their moisture content. When a plate is poured, the surface of the agar is exceedingly moist, and the organisms planted in it grow rapidly till their growth is inhibited by the drying atmosphere of the incubator. Moisture conditions are fairly uniform in ordinary bacteriological technique; colonies of most species are discrete and characteristic. The colonies, when few, are usually pure; the viable aerobes usually all form colonies, and the method as a whole is easy and practical. But with anaerobes the moisture content of the medium and the moisture on its surface become of great importance. I have known agar in deep tubes of medium, which had given perfect results with blackleg colonies, to refuse to give a growth of blackleg when it was somewhat old and dried out, though the agar had nowhere, as yet, separated from the side of the tube. The addition of sterile distilled water made the medium as fertile a soil as fresh agar. I have encountered aerobes which grow to the surface of the agar of a shake, but not in colonies upon its surface. How much more would dryness affect the growth of the more delicate anaerobic organisms on the surface of a plate! Even the hardy tetanus organisms, which grow well in dry deep agar, often refuse to grow on its surface. In order to produce discrete anaerobic colonies plates must be dried after pouring. They must be dried just long enough and not too long. This period varies with the composition, age and thickness of the agar, with the humidity of the atmosphere, and with the moisture present in the anaerobic jar. It takes time and patience to learn to adjust the period for drying the plates. Then when the culture is sown and the plates are ready to incubate, what have we for anaerobic methods? A variety of available atmospheres for the growth of the organisms almost as great as is the number of workers in the anaerobic field: Hydrogen, carbon-

dioxide, nitrogen, illuminating gas, nitrogen-hydrogen-carbon-dioxide and vacuum with varying degrees of moisture, pressure and oxygen present. How can one hope to standardize type colonies under such conditions? And what, may we ask, *is* the proper moisture for the surface of a plate? There is no universal proper moisture. Agar moist enough to grow tetanus will allow the spread of *B. sporogenes* till the *B. sporogenes* has increased a million times more than the tetanus has. Some mixtures of organisms allow isolation of their components by surface methods, and some do not. When discouraged with plates that have dried too long, the worker dries them less, and finds to his joy beautiful discrete colonies, some round and some lobed. He must fish them immediately onto plates or into a deep medium or they may die. But let him beware of a pitfall. Let him hold them to the light without a cover and look between the colonies. A slight film of moisture there may represent a spread of growth which contaminates all his colonies. But such a spread may be difficult or impossible of detection. A fragment of coverslip dropped between colonies may show bacilli. I venture to suggest that it is almost impossible to determine in an agar slant the non-existence of such a thin spread, and such a thin spreading film is far more likely to occur in the confines of a tube than on a plate.

Methods of spreading a culture on a surface do not separate the individual organisms from one another so well as does a shaking in liquid agar—in properly made shakes the colonies are beautifully distributed.

Other minor disadvantages of a surface method are that the plates must be incubated immediately after sowing and be fished immediately after opening; they are usually valueless when reincubated after opening for inspection because of too much drying, and they require the use of more glassware than do deep-tube methods, and also the use of an anaerobic jar or other anaerobic apparatus.

The method of Marino should be recommended for organisms which form minute colonies, and for demonstration plates. Marino poured inoculated agar in the upper half of a Petri dish, and covered it directly with the inverted lower half, and covered the whole with a larger Petri dish. This method is convenient but

not necessary for photographic work, as sections for that purpose may be cut from tubes of agar and may then be mounted between cover and slide. Fehrs and Sachs-Mücke used a similar method, covering the agar with a photographic plate. Krumwiede and Pratt used Marino's method satisfactorily for the isolation of fusiform bacilli, sealing the open crack with wax. Rhein used it with satisfaction for general anaerobic work, pouring a sterile agar layer on either side of the inoculated one. Dick used the method of Rhein, replacing the top dish by a layer of paraffin. All these methods are probably preferable to surface plating for isolation purposes, but are somewhat cumbersome.

Foth complains that the invention of new anaerobic methods has become a sort of sport. Many procedures are too complicated to use, though most methods will serve well for the cultivation of anaerobes. It would seem as though any method employing sticky black pyrogallic acid and alkali should be avoided, or at least only chosen in the modification of Lentz.

Certain workers with surface methods have charged that deep colony procedures do not give pure cultures. Either type of procedure will give pure cultures in the hands of the critical worker and impure ones in the hands of the uncritical one. But I have found in making a large collection of anaerobes that the cultures from laboratories whose isolation procedure was a deep colony method were more often pure than those from laboratories where surface methods were preferred, and I believe that, with the same amount of labor, the same expenditure of time and material, and the same degree of critique, the deep-colony methods are more successful than are surface ones.

Deep-colony methods have been described by the Hesses, by Liborius, and by Veillon and Zuber, and they have been used extensively by von Hibler, Burri, and by French workers. Von Hibler (1908) preferred deep colony isolation to plate methods because of the fact that water of condensation was likely to render plates worthless.

The selection of a suitable medium for deep-colony isolation is an essential to its success. For general work the primary requirement is that the nutriment in the medium allow every anaerobe

present to grow and form a colony. Otherwise colonies may be fished through agar that contains living invisible organisms of other species, and the most deceptive sort of contamination will take place. The medium should be clear and transparent. Our standard agar medium for routine work is made of beef liver. The usual proportion of one part of meat to two of water gave too active a growth and too much gas. The medium is made as follows:

One part of ground beef liver and four parts of distilled water are infused over night, boiled, and strained. To the broth add 1.5 per cent peptone, 0.5 per cent salt, and for ordinary purposes make up with 2 per cent agar pH 7.2 (faintly alkaline to litmus).

When unusually active gas-producers are present, high dilutions and short (twelve hours) incubation periods are resorted to. Such methods always suffice when rapidly growing species are the ones to be isolated. But when slowly growing species are sought in the presence of actively growing ones, other methods are available. To absorb hydrogen, 1 per cent potassium nitrate may be added to the agar (Veillon and Mazé). To prevent the colonies of the rapidly growing types from outrunning the others, use 3 per cent agar or old agar that has partially dried out, or pay particular attention to enrichment of the desired species in the inoculum and employ abundant dilution tubes. Do not depend upon any colony method for the isolation of badly contaminated slowly growing tissue invaders, but resort directly to guinea-pig inoculation. For slowly growing non-pathogenic organisms mixed with rankly growing gas-producers, try a sugar-free agar (von Hibler,1908).

There is an essential point in the employment of deep colony tubes which must be observed. Otherwise the method is of no more use than any other. Actively growing anaerobes frequently leave their colonies and grow in the agar as though it were a broth.

This happens more readily with some types of organisms than with others. *B. Welchii* is the chief offender and should be avoided by heating whenever possible. A tube in which this phenomenon has occurred is readily identified by holding it to the

light with a control. Such tubes are to be regarded as "enrichment cultures." Thus their colonies may be of great use when directly inoculated onto another agar series. They are of no use when inoculated into a liquid medium. The close observation of this phenomenon of "permeating growth" cannot be too earnestly insisted upon.

The deep colonies of anaerobes are highly characteristic. Surface colonies are quite characteristic but are obviously subject to many more outside influences than are deep ones. Often colonies of different strains in the same species are different and sometimes colonies of one type of anaerobe resemble those of an entirely different type. But carefully made agar shakes often give a beautiful picture of the flora of a wound or of a culture. They are very easily observed with a hand lens and may be as closely approached as may surface colonies. Aerobic growth is easily distinguished from anaerobic growth. My routine method of testing for impurity of culture has been to make three dilution shakes on liver agar. The first and second tubes tell whether or not the culture is pure. The third usually furnishes colonies suitable for fishing. I was able to isolate, in two series of three agar tubes each, a strain of *oedematiens* type that had been overgrown 1:500 by a vibrion septique.

Technique of sowing and fishing. Boil the tubes of agar for a minute or two, remove them from the water, shake them, boil them a little longer, shake them again to remove the air, then cool them to 45°. Do not boil them for ten or fifteen minutes or the cotton will become saturated with moisture. For ordinary purposes use three tubes to each culture. For new and important material of doubtful nature or for shyly growing organisms among rankly growing ones, use more tubes. Inoculate tube 1 with one loopful of culture and roll it, tip it, and roll it four or five times. Take a Pasteur pipette[1] of large bore, flame it, draw up agar of tube

[1] It is to be noted that few laboratory workers today understand the making of strong and serviceable Pasteur pipettes, and I hope to be pardoned for describing so simple an operation. Meeker burners are best for this purpose. Heat the glass in the portion of the flame where the heat is nearly uniform for a considerable distance. In a blowpipe or Bunsen flame this is above the cone; in the flame of the Meeker burner it is half an inch above the base. Turn the glass

1, expel it, draw up fresh agar and expel it into tube 2. For cultures containing abundant organisms, give tube 2 2 inches of agar measured in the capillary portion of the tube. For ordinary cultures give 5 inches, for *B. Novyi*, etc., give about two capillaries full. Place the inoculum throughout the length of the agar while withdrawing the pipette, but do not blow air into the agar of tube 2. Roll tube 2. Flame the Pasteur pipette. By means of it place agar from tube 2 in tube 3 to the amount of 0.5 to 1 inch on the upper or thick portion of the Pasteur pipette. Roll the tube. Incubate aerobically at 37°. If actively growing species are present, incubate twelve hours. Otherwise incubate eighteen to twenty-four hours. For blackleg, clostridia, and unknown shy types, incubate four days. Examine the colonies with a hand lens. Look for permeating growth. It is better, in fishing from a tube containing more than one type of colony, to fish once more onto a series of agar tubes. Final isolation should be made from colonies of mixed cultures. Study the tubes carefully with a hand lens, noting minute colonies and aerobic growth. Select the tube to be fished, and, if possible, select the colonies desired. Take a well-made, strong Pasteur pipette of fairly large bore, bend it at right angles where the capillary begins, break the tip, flame the whole capillary. Remove the plug from the tube and loose fibers of cotton from its opening, insert the Pasteur pipette along the side to the bottom, remove and empty it of agar; re-insert it, and blow the whole column of agar into a sterile Petri dish. The large Pasteur pipette may be used many times. One-half Petri dish serves for each tube. Take a short-stemmed Pasteur pipette, hold it in the flame, draw the capillary out to a hair-like tube, and break it off fairly short. Suck up the desired colony and expel it into a tube of meat medium or tube 1 of another agar series. Draw out the

constantly but slowly in the *same* direction, *not* forwards and backwards. Continue till the hot portion softens and contracts to about four-fifths of its former diameter. Never pull the glass while it is in the flame. Remove the rod from the flame and *wait* a second, then pull *slowly*. If the glass is pulled too soon or too quickly the fine bore is formed from the hottest portion only, and not from all the heated glass, the bore is small, and its walls are thin and weak. An hour's continuous practice is necessary to begin with; the art, once learned, is extremely useful and is not forgotten.

pipette again, flaming it well, and use it to isolate two or three more colonies. Other workers employ other methods, which are probably as good. Burri (1902) recommends the use of tubes open at both ends with an autoclaved rubber stopper placed in the lower end. Some use the loop only for purposes of dilution. Some heat the end of the test tube and expel the agar column by force of the steam thus generated. It is necessary to break the tube at the bottom only when an aerobe is present. Burke used a dissecting lens with stand for fishing colonies; Dr. Meyer finds a binocular a great help in some cases. He sections the agar with a sterile blade when researching for minute colonies that are rare. Some workers prefer to attach a rubber tube or a teat to the pipette used in fishing. It is theoretically wrong to fish the colonies from the top of the column of agar without removing it from the tube, because the capillary may pass ungerminated organisms, but such a method might prove practical when used with discretion. Some workers fish the colonies with a platinum needle, but this would hardly prove as satisfactory as a pipette method.

Methods of single-bacillus isolation. Isolation of a single bacillus has been resorted to for the separation of anaerobes. Miss Robertson found that the India-ink method of Burri (Besson 1913) exposed the organisms too much and they failed to germinate. I used the Barber method for some time for blackleg and vibrion-septique organisms, and found that the exposure killed vegetative forms and that spores were necessary to give a growth. I fished from apparently pure cultures various numbers of organisms, from one to ten, into meat tubes and used for a type strain the tube that grew and had received the fewest bacilli. I found the method wasteful of time, material, eyesight, and nervous energy, and have abandoned it. My employment of the apparatus was, however, far from being as skillful as that of Dr. Barber. I explained my difficulties to Dr. Barber and he (1920) has made a careful statistical study of the behavior of various anaerobes when isolated by his technique. He was successful when inoculating various media with different anaerobes in securing 62 growths from 400 single bacilli, and 93 growths from 211 single spores. Vegetative rods

of vibrion-septique were particularly sensitive to the air. Barber found the semi-solid medium of Lignières excellent for securing growth of single anaerobes. Colonies could be secured from spores in a chamber containing a *Pseudomonas pyocyanea* culture.

Malone and Holker have devised pipette methods for single bacillus isolation with which I have no personal experience. It may be that they protect the organisms from air better than does the Barber method. Hecker makes interesting technical suggestions. Hort objects to all methods of single bacillus isolation from liquids and he objects to capillary methods on account of optical difficulties. The method preferred by Hort, the perforated plate method, is too aerobic for our purposes.

IX. *Animal inoculation* is frequently resorted to for the isolation of anaerobes. The guinea-pig is the best animal for this purpose. It is highly susceptible to infections and also develops very characteristic lesions, of diagnostic value. Animal inoculation is of prime value for recovering pathogens that have been badly overgrown. It is the only way I know of to recover blackleg organisms that have been grossly contaminated. A drop of lactic acid may be used for a second trial, if the first fails. Inoculation of mixtures from the involved tissues of gas-gangrene cases is of course necessary, but it is likely to give misleading ideas as to the flora of those tissues. It is advisable to run deep-colony cultures from various portions of an amputated limb or of material derived from tissue-puncture or from a blood culture, and to inspect the flora of the limb carefully. A culture or smear from the wound itself may give very misleading data as to the etiology of a gangrenous process. I believe that the rôle played by *B. Welchii* in gas-gangrene nfections has been grossly exaggerated because of the failure of many workers to study carefully the flora from various portions of infected muscle. When the colony method has given pure or apparently pure cultures, inoculate them into guinea-pigs and record results. When immediate identification of pathogens is urgent, examine smears made by puncture from affected tissue *remote* from the wound. Conjecture the possible types there

represented, inoculate a series of guinea-pigs with antitoxic or antibacterial sera of the groups probably represented on the smears, in such a manner that for each type of organism there is a guinea-pig immunized against the other types only. Then inoculate the mixed material into all the guinea-pigs. This method was found successful by the Committee. In large war hospitals collections of guinea-pigs immunized by bacterial inoculation have been kept for diagnostic purposes.

It is best to inoculate guinea-pigs in the thigh muscles. Take cultures from various points in the body. The heart-blood culture is usually the most valuable. *Oedematiens*-group organisms and some other pathogens do not always become septicemic, however. Bifermentans-group organisms and other proteolytic types may become septicemic. Inoculate into another guinea-pig a culture from the heart-blood in smaller quantity than was used before. If this fails, isolate the proteolytic organism, immunize a guinea-pig with it, then inoculate the mixture. For all animal work keep a careful record of the cultures inoculated, incubation periods, lesions in the animals, and, above all, make constant use of the microscope.

Anaerobic organisms should be sought in the following pathological conditions:

Infected wounds (rods or cocci).
Gangrene.
Oedema.
Emphysema of muscles, connective-tissue, liver or other organs.
Haemorrhagic condition of muscles.
Pneumonic processes where anaerobic infection is suspected, pulmonary gangrene.
Necrosis of muscle or connective tissue (*B. necrophorus et alii*).
Injection of serous surfaces, especially in ruminants.
Abortion in animals (search foetus for *Bact. abortum*).
Endometritis, post abortum or post partum (*Streptococcus*).
Appendicitis and various ulcerative and suppurative conditions.
Tetanus (in absence of wounds and uterine infection, search for peridental infection).
Botulism, intestinal content and wall, liver, spleen, stools from patients.

Obscure fevers, measles, scarlet fever. Blood cultures, look for various invaders.
Rhinitis, Vincent's angina; mucous surfaces.

Make smears of affected tissue, make meat or brain cultures and make at the same time numerous shakes in deep liver-agar. Examine shakes twelve hours after incubation if possible, and examine meat cultures twenty to twenty-four hours after incubation. Blood cultures in broth, meat or agar should always be made, if possible, *ante mortem* and *post mortem*.

I wish to express my thanks to Dr. Karl F. Meyer for his active interest and coöperation in this work.

SUMMARY

As an aid to the isolation of anaerobes the following notes may be observed:

1. Success in the isolation of anaerobes depends more on the critical sense of the worker than on the method he employs.

2. Microscopic observation should be made of incoming material and of cultures after twenty-four and forty-eight hours' incubation, and the development of a critical eye for the morphology and staining reactions of anaerobes is imperative.

3. Heating of material should be executed according to the logical requirements for that material. Heating at 70° in pipettes is to be recommended for routine work.

4. A routine medium should be employed which will favor as many diverse forms as possible. Chopped beef heart, preferably containing a little peptic digest broth, the reaction at about pH 7.2, presents numerous advantages as a routine medium, for most of the anaerobes studied in a pathological laboratory. If freshly boiled it is usually quite unnecessary to incubate it anaerobically.

5. Selective media may be employed for special purposes, and they offer many possibilities.

6. Isolation by means of guinea-pig inoculation, securing the organism from the heart's blood or from the affected tissues remote from the site of inoculation, is preferable for invading patho-

gens, but may not be depended upon to give a true picture of the pathogenic flora of the material injected.

7. The making of dilution shakes in deep agar (method of Liborius and of Veillon) is to be preferred to other colony methods; care must be taken to isolate for a type a colony from an apparently pure culture.

8. A medium for dilution shakes should afford an opportunity for growth to just as many species as possible. Such a medium is pepton-liver agar, as described in the text.

9. When once pure, a culture should be carefully kept pure. Re-incubation, prolonged incubation in closed jars, storing in closed cans or in dusty places, are to be avoided. Autoclaved media only should be employed for the preservation of type cultures; one cannot be too careful as to routine technique.

REFERENCES

ACHALME-PASSINI See Choukevitch (1911).

ANKERSCHMITT, P. 1905 Untersuchungen über die Bakterien im Verdauungskanal des Rindes. Centralbl. f. Bakteriol., O., **39**, 359, 574, 687; Ibid., **40**, 100.

BARBER, M. A. 1914 The pipette method in the isolation of single microorganisms and in the inoculation of substances into living cells. Philippine Jour. of Science, **19**, 307.

BARBER, M. A. 1920 The use of the single cell method in obtaining pure cultures of anaerobes. Jour. Exper. Med., **32**, 295.

BERGEY, D. H. 1919 Thermophilic bacteria. Jour. Bacteriol., **4**, 301.

BESSON, A. 1913 Practical bacteriology, microbiology and serumtherapy. London.

BREDEMANN, G. 1909 Bacillus amylobacter A. M. et Bredemann, in morphologischer, physiologischer und systematischer Beziehung. Centralbl. f. Bakt. II. Abt., **23**, 385.

BURKE, G. S. 1919 Notes on Bacillus botulinus. Jour. Bact., **4**, 555.

BURRI, R. See Besson 1913.

BURRI, R. 1902 Zur Isolierung der Anaëroben. Centralbl. f. Bakteriol. Abt. II., **8**, 533.

CHOUKEVITCH, J. 1911 Étude de la flore bactérienne du gros intestin du cheval. Ann. de l'Inst. Past., **25**, 247.

CHURCHMAN 1912 The selective bactericidal action of gentian violet. Jour. Exper. Med., **16**, 221.

COMMITTEE on Anaerobic Infections: Medical Research Committee, Reports of the Committee upon Anaerobic Bacteria and Infections. Report on the anaerobic infections of wounds and the bacteriological and serological problems arising therefrom. 1919. Special Report Series, No. 39, London.

DICK, G. F. 1918 A plate method for isolating anaerobes. Jour. Inf. Dis., **23**, 578.

DICK, G. F., AND HENRY, G. R. 1914 Anaerobic cultures in scarlet fever. Jour. Inf. Dis., **15**, 85.

FEHRS, AND SACHS-MÜCKE 1908 Beitrag zur Züchtung und Isolierung von Anaerobiern. Centralbl. f. Bakteriol. I. O., **48**, 122.

FOTH 1909 Die Diagnose des Rauschbrandes. Zeitschr. f. Infekkh. d. Haustierre. **6**, 201.

FRED, E. B. 1916 A laboratory manual of soil bacteriology. Philadelphia and London.

FRIBES See Winogradsky (1895).

GHON, A., AND SACHS, M. 1902 Beiträge zur Kenntnis der anaeroben Bakterien des Menschen. Ueber die anaerobe Züchtung. Centrabl. f. Bakteriol., **32**, 403.

HALL, I. C. 1919 Selective elimination of hay bacillus from cultures of obligative anaerobes. Jour. Am. Med. Assoc., **72**, 274.

HALL, I. C. 1920 Method for purification of obligate anaerobes. J. Inf. Dis., **27**, 576.

HECKER, F. 1916 A new model of double pipette holder and the technic for the isolation of living organisms. J. Inf. Dis., **19**, 306.

HENRY, H. 1917 A simple device for the growth of anaerobes on plates. Brit. Med. J., **1**, 762.

HESSE, W., AND HESSE, R. 1885 Ueber Züchtung der Bazillen des malignen Oedems. Deutsche med. Wchnschr., **11**, 214.

VON HIBLER, E. 1908 Untersuchungen über die pathogenen Anaeroben. Jena.

HOLKER, J. 1919 Micro-and macro-methods of cultivating anaerobic organisms. J. Path. and Bacteriol., **22**, 28.

HORT, E. C. 1920 The cultivation of anaerobic bacteria from single cells. J. Hyg., **18**, 361.

KITASATO, S., UND WEYL, TH. 1890 Zur Kentniss der Anaëroben. Zeitschr. f. Hyg., **8**, 41.

KRUMWIEDE, C., AND PRATT, J. 1913 Isolation and cultivation of fusiform bacilli. J. Inf. Dis., **12**, 199.

KÜSTER, E. 1913 Kultur der Mikroorganismen. Leipzig and Berlin.

LENTZ, O. 1910 Ein neues Verfahren für die Anaerobenzüchtung. Centralbl. f. Bakteriol. I. O., 1910, **53**, 358.

LENTZ, O. 1919 Friedberger und Pfeiffer. Lehrbuch der Mikrobiologie. Jena. p. 370.

LEWKOWICZ, X. 1906 Ueber die Reinkulturen des fusiformen Bacillus. Centrabl. f. Bakteriol. I., **41**, 153.

LIBORIUS, P. 1886 Beiträge zur Kentnis des Sauerstoffbedürfnisses der Bakterien. Zeitschr. f. Hyg. u. Infekkh., **1**, 115.

LIGNIÈRES, J. 1919a Nouvelle méthode pour la recherche et la culture des anaerobies pouvant servir au diagnostic des affections causees par les microbes. Bull. Acad. Med., 3, s., **82**, 181.

LIGNIÈRES, J. 1919b Nouvelle méthode très simple pour cultiver facrlement les microbes anaérobies. Les millieux semi-liquides en bactériologie Compt. rend. Soc. Biol., **82**, 1091.

MALONE, R. H. 1918–1919 A simple apparatus for isolating single microorganisms. J. Path. and Bacteriol., **22**, 222.

MARINO, F. 1907 Methode pour isoler les anaérobies. Ann. de l'Inst. Past., **21**, 1005.

MCCOY, G. W., AND BENGTSON, I. A. 1919 Enrichment described. Am. J. Public Health, **9**, 427.

MCINTOSH, J., AND FILDES, P. 1917 The classification and study of the anaerobic bacteria of war wounds. Med. Res. Comm. Special Report Series No. 12, London.

NORTHRUP, Z. 1916 A simple apparatus for isolating anaerobes. J. Bact., **1**, 90.

OMELIANSKY, W. L. 1895 Sur la fermentation de la cellulose. Compt. rend. Soc. Biol., **121**, 653.

OMELIANSKY, W. L. 1899 Ueber die Isolierung der Nitrifikations-mikroben aus dem Erdboden. Centralbl. f. Bakteriol. Abt. II., **5**, 537.

OMELIANSKY, W. L. 1902 Ueber die Gärung der Cellulose. Ibid., **81**, 226.

OMELIANSKY, W. L. 1904 Ueber die Trennung der Wasserstoff und Methangährung der Cellulose. Ibid. **11**, 369, 703.

PFUHL, E. 1907 Die Züchtung anaërober Bakterien in Leberbouillon, sowie in Zuckerbouillon, etc. Centralbl. f. Bakteriol. I. O., **44**, 378.

PLOTZ, OLITZKY, AND BAEHR 1915 The etiology of typhus exenthematicus. J. Inf. Dis., **17**, 1.

RHEIN, M. 1919 La culture et l'isolement des anaérobies sans appariellage spécial. Le Presse Med., **27**, 504.

RIVAS, D. 1902 Ein Beitrag zur Anaërobenzüchtung. Centralbl. f. Bakteriol., **32**, 831.

SCHOTTMÜLLER, H. 1910 Zur Bedeutung einiger Anaeroben. Mitt. a.d. Grenzgebiete d. Med. u. Chir., **21**, 450.

SHOUTEN, S. L. 1905; 1907 Reinkulturen aus einen unter dem Mikroskop isolierten Zelle. Zeitschr. f. wiss. Mikr., **22**, 10; **24**, 258. See also Küster, 57. 1913.

STICKEL, J., AND MEYER, K. F. 1918 Inexpensive culture mediums. J. Inf. Dis., **23**, 68.

STODDARD, J. L. 1918 Points in the technic of separating anaerobes. J. Am. Med. Assn., **79**, 906.

STURGES, W. S., AND RETTGER, L. F. 1919 Isolation and cultivation of *B. putrificus*. J. Bact., **4**, 171.

TULLOCH, W. J. 1917 On the bacteriology of wound infections in cases of tetanus and the identification of *B. tetani* by serological reactions. J. Roy. Army Med. Corps. **29**, 631.

TULLOCH, W. J. 1919 The isolation and seriological differentiation of *B. tetani*. Proc. Roy. Soc., B 90, 145.

TUNNICLIFFE, R. 1913 An anaerobic organism associated with acute rhinitis. J. Inf. Dis., **13**, 283.

TUNNICLIFFE, R. 1917 The cultivation of micrococcus from blood in pre-eruptive and eruptive stages of measles. J. Am. Med. Assn., **68**, 1029.

VEILLON, A., AND MAZÉ, P. 1910 De l'emploi des nitrates pour la culture et l'isolement des microbes anaérobies. Compt. rend. Soc. Biol., **68**, 112.

VEILLON, A. AND ZUBER 1898 Recherches sur quelques microbes strictement anaérobies et leur role en pathologie. Arch. de Med. Exper., 1st, ser. **10**, 516.

WILSON, J., AND STEER, P. 1918 Points in the technivue employed in the isolation and cultivation of anaerobic bacteria. Brit. Med. J., **2**, 568.

WINOGRADSKY, S. 1893; 1894 Sur l'assimilation de l'azote gazeux de l'atmosphère par les microbes. Compt. Rend. Acad. Sciences, **66**, 1385; **68**, 353. 1894 Centralbl. f. Bakteriol., **16**, 129.

WINOGRADSKY, S. 1895 Sur le rouissage du lin et son agent microbien. Compt. Rend. Acad. Sciences, **71**, 742.

WINOGRADSKY, S. 1902 Clostridium Pastorianum, seine Morphologie, und seine Eigenschaften als Buttersäureferment. Centralbl. f. Bakteriol. II, **9**, 49.

ZEISSLER, J. 1918 Die Pferdeblut-oder Schafblut-Traubenzuckeragarplatte als Ersatz für die Menschenbluttraubenzuckeragarplatte zur Züchtung der pathogenen anaerobier. Deutsche. med. Wchnschr., **64**, 942.

INDOL PRODUCTION BY BACTERIA

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The formation of indol in certain culture media has long been considered an important differential characteristic for the idenification of bacteria. Special interest in the test has been recently aroused in connection with investigations on respiratory diseases because Pfeiffer's bacillus appears to be practically the only mouth organism producing indol (Jordan, 1919, Malone, 1920); in consequence of which Malone has suggested that the test be used as an index of the presence of this organism without actually isolating it. Rivers (1920) has made a similar suggestion for diagnosis of influenzal meningitis. For this paper we have attempted to collect from the literature the more recent information, both positive and negative, concerning indol production by bacteria. On account of the uncertainty of indol tests much of the older work is unreliable. This information has been supplemented by tests on over 180 strains of bacteria, most of which are being carried as stock cultures in this laboratory and have been collected from a great variety of sources. Incidentally we have made a comprehensive comparison of three recommended media and also determined the effect of the incubation period on indol production.

MEDIA

Indol is a disintegration product of proteins containing the tryptophane group. The ideal medium would therefore be one to which pure trytophane had been added but on account of the difficulty of obtaining the material it is impractical, although Zipfel (1913) used such a medium. Dunham's pepton is the easiest medium to prepare and has been most frequently used.

Sicre (1909) and Porcher (1911) have studied the use of various peptons finding that some already contain indol and that with others indol is never produced by bacteria, so that it is necessary not only to make blank tests on any chosen medium but also to control experiments with a known indol-producing organism. In each instance our tests were controlled by inoculating one tube of medium with *Bact. coli*, an indol-forming organism, and another with *Bact. typhosum*, a non-idol-former, and we regarded such controls as essential. For media, besides Dunham's pepton and the tryptophane medium of Zipfel, Rivas has suggested trypsinized pepton (Rivas, 1912) and Cannon (1916), instead of preparing pure tryptophane from casein as was done by Zipfel, used hydrolyzed casein as the basis for his medium.

Homer (1916) believes that tryptophane is necessary for bacterial life and if not present will be synthesized by the organisms. Logie (1920) has used synthetic media containing ammonium lactate with asparagin or sodium asparaginate and claims that indol producing organisms possess an enzyme which enables them to split off and utilize part of the tryptophane molecule. It is possible that many organisms may be capable of synthesizing indol but make use of it in their metabolism.

For the hemophilic group a heated blood broth has been used (Jordan, 1919).

It is well known that the addition of glucose to a medium interferes with the indol test. Fischer (1915) reports that lactose, galactose, maltose or fructose are without effect. He believes that this action of glucose is due not to acid production, as had been supposed, but to the inactivation of the proteolytic enzyme concerned in splitting the tryptophane. Logie (1920) found that if glucose was added to a living culture of *Bact. coli* in which indol had already been produced the latter rapidly disappeared. From this he concluded that glucose caused an increased demand in the organism for indol. Homer (1916) explains the effect of the presence of glucose either on the basis of the preference of the organism for glucose over tryptophane or by assuming the formation of a chemical compound between glucose and tryptophane which is relatively stable.

We have used three media. (1) Dunham's pepton solution. One per cent of pepton (Armour's) and 0.5 per cent sodium chloride were dissolved in distilled water and the reaction adjusted to +1 to phenolphthalein. (2) Rivas' trypsinized pepton (Rivas 1912). Ten grams of pepton (Armour's) were dissolved in 200 cc. distilled water. To this was added a solution of 0.5 gram trypsin in 10 cc. of water (trypsin dissolved by shaking and gentle heating not over 40°C.) and digestion allowed to continue for three hours at 37°C., with frequent stirring. The solution was then made up to 1 liter and reaction adjusted to + 1 to phenolphthalein. (3) Cannon's casein medium (Cannon, 1916). Twenty grams of chemically pure casein were added to 250 cc. distilled water and the whole made alkaline to phenolphthalein with sodium carbonate. One-half gram of trypsin was added and the casein allowed to digest for six hours. The medium was then autoclaved and 5 grams each of asparagin and ammonium lactate, 2 grams of dipotassium phosphate and 0.2 gram magnesium sulphate were added. The solution was made up to 1 liter and reaction adjusted to +1 to phenolphthalein.

A large number of comparative tests were made on these three media. In no case did the final indol test vary but a positive reaction was obtained more quickly, and the color tests were stronger, with the trypsinized casein or pepton than with Dunham's pepton solution. Positive tests with the trypsinized pepton were noted after six hours incubation with *Bact. coli* and color production with Ehrlich's reagent was at its maximum at the end of twenty-four hours. With Dunham's solution the maximum was obtained only after four days. After six days the indol began to disappear. As most of our tests were made simultaneously on all three media we used the four day period, although forty-eight hours is sufficient for the trypsinized media.

The influence of oxygen supply on indol formation has been studied by Porcher and Panisset (1911). They found that growing cultures of the colon bacillus and of proteus anaerobically decreased the amount of indol formed, while if a current of oxygen was kept going through the flask, the amount was increased. However, they were unable to provoke the formation

of indol by *Bact. typhosum* by an oxygen current. Our cultures were incubated aerobically, except in the case of the strict anaerobes.

INDOL REAGENTS

Numerous tests for indol have been suggested and used. Nelson (1916) gives four: (1) dimethylamine, glycolic acid, glyceric aldehyde and sulphuric acid, giving a pink color; (2) peruvic aldehyde, sulphuric acid and ferric sulphate, giving a violet color; (3) vanillin and an acid, giving an orange color; (4) Salkowski test—sulphuric acid and potassium nitrite, giving a pink to red ring. Escallon (1908) recommends furfural. This, in the presence of hydrochloric acid, gives an orange yellow color. It is claimed that this test is sensitive to 1 part in 800,000. Baudisch (1915) describes a reaction using nitromethane. By far the most satisfactory test is that suggested by Ehrlich (1901). The reagent is prepared by dissolving 4 grams of paradimethylamido-benzaldehyde in 380 cc. of alcohol and adding 80 cc. of concentrated hydrochloric acid. A red color is formed in the presence of indol at the junction of the reagent and the liquid to be tested if the former is added so that it forms a layer on top. A solution of potassium persulphate is sometimes added to bring out the color more clearly but we have found the reagent quite satisfactory without this. In making our tests, if a red color appeared on adding the Ehrlich reagent, 1 cc. of amyl alcohol was added and the tube shaken. The red coloring matter, if due to indol, is soluble in amyl alcohol.

SUMMARY

In the following table we have summarized the results of our tests together with those we have been able to find in the literature. Owing to the uncertainty of results obtained by use of the older methods of testing for indol, only relatively recent work has been included. The organisms are divided into two groups: (I) those which may pretty definitely be regarded as giving negative tests and (II) those for which positive results have been reported. It should be noted that in every instance where

any considerable number of strains of an organism in group II have been examined, negative as well as positive results have been reported, with the exception of the cholera vibrio.

From this summary we must conclude that whereas the indol test may serve as a valuable aid in differentiating bacteria, it cannot be regarded as an absolute criterion. A positive test may give definite information but a negative test must be interpreted with caution.

We should also like to emphasize the necessity for a standard reagent for the indol test and suggest the use of Ehrlich's dimethylamidobenzaldehyde solution for this purpose.

In this table we have used the classification adopted by the Society of American Bacteriologists (Winslow, 1920).

Group I. Indol negative

Bcaillus anthracis* (Zipfel)
subtilis*
Actinomyces asteroides*
bovis*
graminaris*
Bacterium abortum (Weeter)
cloacae (5 strains*, Kligler)
enteritidis (3 strains*, Crossonini, Porcher and Panisset, Zipfel, Nonnotte and Demanche)
fecalis-alcaligenes (3 strains*)
icteroides (Crossonini)
mucosum-capsulatum* (Hiss and Zinsser)
paratyphosum A (2 strains*, Zipfel, Jordan, Nonnotte and Demanche)
paratyphosum B (13 strains*, Zipfel, Jordan, Nonnotte and Demanche)
pullorum (5 strains*, Mulsow)
rhinoscleromatis*
sanguinarium (Mulsow)
suipestifer (4 strains*, Zipfel, Crossonini)
typhosum (15 strains*, all investigators)
typhi-murium (Nonnotte and Demanche)
Clostridium botulinum (6 strains*)
chauvei*
Welchii*
Corynebacterium Hoffmannii (3 stains*)
pseudodiphtheriae (4 strains*)
xerosis (2 strains*)

* Our tests.

Diphtheroids (Malone)
Diplococcus pneumoniae (Jordan, Malone)
Erythrobacillus miniaceus*
mycoides-roseus*
mycoides-corallinus*
prodigiosus (4 strains*, Crossonini, Zipfel)
Myobacterium leprae*
Moelleri (2 strains*)
tuberculosis (4 strains*, Zipfel)
Neisseria meningitidis (Jordan)
catarrhalis (Malone)
Pseudomonas cyanea*
cyanogenes*
violacea*
Pasteurella pestis* (Zipfel)
Staphlococcus albus (8 strains*, Zipfel)
aureus (4 strains*, Zipfel)
citreus (Zipfel)
not specified (Jordan, Malone)
Streptococcus viridans (13 strains*, Jordan, Malone)
hemolyticus (2 strains*, Zipfel)
rheumaticus*
Sarcina lutea*
rosea*
Vibrio tyrogenus (Deneke)* (Zipfel, Tobey)
Zopfius zopfii (3 strains*)
Sporothrix schenkii*
Blastomycetes dermatitidis*
Sac. pastorianus*

Group II. Indol positive or negative

Bacterium aerogenes* (Kligler, Chen and Rettger)†
coli* (Kligler, Chen and Rettger)‡
dysenteriae* (Zipfel, Kolle and Wassermann)§
Clostridium sporogenes (edematis)* (Bertrand)
tetani (Hall)
Corynebacterium diphtheriae (Escallon, Zipfel)
Hemophilus influenzae (Rhein, Jordan, Malone)±

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* Our tests.

† Chen and Rettger found 141 strains +, 306 −.

‡ Chen and Rettger found from feces 173 strains +, 0 −; from soil 15 strains + 5 −.

§ We found as + Flexner, Hiss-Russel, Shiga, 110, 12 U. S., as − Hoffmanni, 177. Zipfel found Flexner andY +, Shiga −. Kolle and Wassermann give Strong −.

± Rhein found 7 strains +, 1 −; Jordan 18 +, 7 −; Malone found 92 per cent +, 8 per cent −.

Pasteurella aviseptica (Mulsow, Kolle and Wassermann)
Proteus group (Bengston, Horowitz, Kligler, Larson and Bell, Rhein, Sicre)
Pseudomonas pyocyanea* (Jordan)¶
Vibrio cholerae (2 strains*, Crossonini, Baudisch, Zipfel, Tobey)
finklerei (Crossonini, Tobey, Zipfel)
metschnikovi* (Crossonini, Steensma, Tobey)
protea*

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* Our tests.

¶ We found 13 strains −, 6 of them freshly isolated; Jordan reported both + and −; see also Lartigau (1898)

REFERENCES

Baudisch 1915 Über eine neue Indolreaktion, Zeitschr. Physiol. Chem., **94**, 132.

Bengston 1919 The proteus group. Jour. Infect. Dis., **24**, 445.

Bertrand 1913 Influence des régime alimentaire sur la formation de l'indol dans l'organisme. Ann. Inst. Pasteur, **27**, 76.

Cannon 1916 A simple and rapid indol test. Jour. Bact., **1**, 535.

Chen and Rettger 1920 A correlation study of the colon-aerogenes group of bacteria, with special reference to the organisms occurring in the soil. Jour. Bact., **5**, 253.

Crossonini 1910 Über den Nachweis von Indol. Arch. Hyg., **72**, 161.

Ehrlich 1901 Über die Dimethyl-Amidobenzaldehyde Reaction. Berl. Med. Wochensch., **1**, 151

Escallon 1908 Reserche de l'indol dans les cultures microbiennes a l'aide du furfural. Compt. rend. soc. biol., **65**, 507.

Fischer 1915 Hemmung der Indolbildung bei *Bact. coli* in Kulturen mit Zuckerzusatz. Biochem. Zeitschr., **70**, 105.

Hall 1920 Unpublished communication.

Hiss and Zinsser 1917 Textbook of Bacteriology.

Homer 1916 A suggestion as to the cause of lessened production of indol in media containing glucose. Jour. Hygiene, **15**, 401.

Horowitz 1916 Contribution a l'étude du genre Proteus Vulgaris. Ann. Inst. Pasteur, **30**, 307.

Jordan 1899 Bacillus pyocyaneus and its pigments. Jour. Exp. Med., **4**, 627.

Jordan 1917 Differentiation of the paratyphoid-enteriditis group I. Jour. Infect. Dis., **20**, 7.

Jordan 1919 Production of indol by strains of Pfeiffer's bacilli. Jour. Am. Med. Assn., **72**, 1542.

Jordan 1920 Biology of the Pfeiffer bacillus. Am. Jour. Pub. Health, **10**, 648.

Kligler 1914 Observation on indol production in the Colon-typhoid group. Jour. Infect. Dis., **14**, 81.

Kolle and Wassermann 1913 Handb. d. pathogen. Mikroorganismen.

LARSON AND BELL 1913 A study of the lesions produced by Bacillus proteus. Jour. Infect. Dis., **13**, 510.

LARTIGAU 1898 Epidemic dysentery caused by Bacillus pyocyaneus. Jour. Exp. Med., **3**, 604.

LOGHEM AND LOGHEM-POUW. 1912 Beiträge zur Differenzierung des Proteus Groupe. Centrabl. Bakt., Orig., I. Abt., **66**, 19.

LOGIE 1920 Synthesis of tryptophane by certain bacteria and the nature of indol formation. Jour. Path. and Bact., **23**, 224.

MALONE 1920 The production of indol by Pfeiffer's bacillus. Indian Jour. Med. Res., **7**, 519.

MULSOW 1919 Avian paratyphoid bacilli. Jour. Infect. Dis., **25**, 135.

NELSON 1916 Some color reactions for indole and scatole. Jour. Biol. Chem., **24**, 527.

NONNOTTE AND DEMANCHE 1908 Sur la recherche de l'indol dans les cultures microbiennes. Compt. rend. soc. biol., **64**, 658.

PORCHER 1911 Les diverses peptone et la formation de l'indol. Compt. rend. soc. biol., **70**, 464.

PORCHER AND PANISSET 1909 Indol dans les bouillons microbiens. La présence dans las cultures du cholera des poules. Compt. rend. soc. biol., **66**, 624.

PORCHER AND PANISSET 1911 La formation de l'indol dans les cultures en milien aerobes et en milien anaerobes. Compt. rend. soc. biol., **70**, 436.

RHEIN 1919 Sur la production d'indol per le bacille de Pfeiffer. Compt. rend. soc. biol., **82**, 138.

RIVAS 1912 Studies on indol. Centralbl. Bakt., Orig., I. Abt., **63**, 547.

RIVERS 1920 Indol test on the spinal fluid for rapid diagnosis of influenzal meningitis. J. Am. Med. Assn., **75**, 1495.

SICRE 1909 Sur la reserche de l'indol dans les cultures microbiennes a l'aide des nouveaux réactife. Compt. rend. soc. biol., **67**, 76.

STEENSMA 1906 Über den Nachweis von Indol und die Bildung von Indol vortausehenden Soffen in Bakterien Kulturen. Centralbl. Bakt., Orig., I. Abt., **41**, 295.

TOBEY 1906 Cholera red and indol reaction. Jour. Med. Res., **15**, 305.

WEETER 1920 Unpublished communication.

ZIPFEL 1913 Weitere Beiträge zur Kenntniss der Indolreaktion. Centralbl. Bakt., Orig., I. Abt., **67**, 583.

WINSLOW AND OTHERS 1920 The Families and Genera of the Bacteria. Jour. Bact., **5**, 191.

ON NITRIFICATION

IV. THE CARBON AND NITROGEN RELATIONS OF THE NITRITE FERMENT

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Winogradsky (1890) showed that the organism of nitrosofermentation is capable of growing in a medium completely void of fixed organic matter when in presence of ordinary air. He proved, thereby, that the organism must derive its carbon from one or all of the following sources; the mineral carbonates, the free carbon dioxide, or the volatile organic compounds of the atmosphere.

That a source of carbon is found and utilized by the organism is supported by the fact that analysis of the culture liquid after nitrosofermentation has taken place shows it to contain an appreciably greater quantity of carbon than it did before the fermentation was activated by inoculation.

Godlewsky (1892, 1895) as well as Winogradsky and Omeliansky (1899), found that cultures of nitrite and nitrate-forming organisms lacking free or combined carbon dioxide could not develop. In discussing the experiments of Winogradsky, above referred to, Beijerinck (1903), points to the possibility that the carbon was not fixed by the cells but by the magnesium oxide present in the basic carbonate used by this investigator. If this criticism were true it is hard to understand why the ratio of "nitrogen nitrified" to "carbon assimilated" should be a constant value. It might be assumed that the results of Ashby (1907–8), (soon to be related) could support Beijerinck's hypothesis, but close scrutiny shows them to bear a different significance.

It appears, therefore, that the function of nitrosofermentation is closely and intimately connected with the function of carbon assimilation by the bacterial cells. In fact, from the data at our disposal it appears as if their separation were impossible. Loew (1891) as early as 1891, soon after the classical researches of Winogradsky, advanced the view that the process of nitrosofermentation does not take place according to the path described in the accompanying equation:

$$2\ NH_3 + 3O_2 \rightarrow 2NO_2H + 2H_2O$$

but rather by the path symbolized as follows:

$$2\ NH_3 + 2O_2 \rightarrow 2NO_2H + 4H$$

The hydrogen liberated during the reaction is not found free in the medium but is momentarily formed in the cells and utilized in a synthetic process according to the following equation:

$$CO_2 + 4\ H \rightarrow CH_2O + H_2O$$

The resulting formaldehyde is not condensed into carbohydrate but directly into protein substances. The fact that nitrates are not utilized by the organism is cited by Loew in support of the above view.

From a physiological standpoint the question is an important one, and a full understanding of its "*modus operandi*" will lead to an understanding of the formation of the oxidized nitrogenous compounds in nature and of the early phases of the accumulation of the carbon supplies on the earth.

EXPERIMENTAL

It was at first necessary to establish whether the organism from American soils behaved towards carbon dioxide in a manner similar to that of the organisms from Europe and Asia, since the differences in form and life-cycle shown by these different organisms could well be accompanied by differences in physiological characteristics. With this aim in view, in 1914 a soil which had received the application of 9000 pounds of calcium carbonate

per acre was used. Three 100 gram lots in the air-dry condition were each moistened with 20 cc. of a 0.5 per cent solution of ammonium sulphate and incubated at room temperature under the following conditions: no. 1, together with a beaker of water, under a bell-jar sealed with vaseline to a glass plate; no. 2, under a bell-jar together with a beaker of soda-lime; no. 3, in the open laboratory air. After thirty days the soils were extracted with water, and the nitrates determined in the extract by means of the phenoldisulphonic acid method. The results obtained are summarized in table 1.

TABLE 1

NUMBER	CONDITION OF ATMOSPHERE	NITROGEN AS NITRATES PER KILO OF SOIL		
		At the start	At the end	Formed
		mgm.	mgm.	mgm.
1	Natural but limited	15	168	153
2	Carbon dioxide removed	15	58	43
3	Natural open	15	125	110

It thus appears that the process of nitrification in American soils is appreciably hindered by the removal of the free carbon dioxide. At first it would seem as if these results were contrary to those given by Godlewsky, since even in presence of soda lime a certain amount of nitrification did take place. Nevertheless, the arrangement of the experiment may account for this. The soil was in Erlenmeyer flasks and the bell-jars used were of 5000 cc. capacity. Had the soda-lime been in the immediate vicinity of the soil, the carbon dioxide removal would have been more complete. Furthermore, in a soil containing organic matter, decompositions are always taking place, and by virtue of the great surface and high retentive power of soils for gases, enough carbon dioxide may easily have been retained to furnish a source of this gas in the immediate vicinity of the bacterial cells. Using the ratio N/C = 36 established by Winogradsky, the quantity of carbon fixed in the nitrification of 4.3 mgm. of nitrogen, would be 0.1 mgm. a quantity easily retained by the soil.

Indications are, therefore, that the organism of nitrosofermentation acting in the soils with which the present work was undertaken, behaves towards free carbon dioxide in a manner similar to the organisms with which Winogradsky and Godlewsky were working. Additional proof on this point is furnished by experiments in solutions in which the above disturbing factors were eliminated.

Experiment 244

In each of two large flat bottomed Fernbach flasks were placed 50 cc. of the ordinary Omeliansky solution, and both were inoculated with an active culture of *Nitrosococcus* from Wooster soil, in equal amounts. One was incubated with no additional treatment while a small container of concentrated potassium hydroxide was placed in the neck of the other and the flask sealed with a manometer tube so as to avoid negative pressures in the system. After incubation at 25°C. for fifteen days the following results were obtained.

TABLE 2

NUMBER	TREATMENT	NITRITE NITROGEN FORMED
		mgm.
1	Normal air	16.98
2	Air minus CO_2	0.41

There is evidently no doubt that the organisms acting in Wooster soil are physiologically similar to those acting in European soils.

A system containing the ordinary Omeliansky solution for nitrite formation from ammonium sulphate, and magnesium carbonate as a base, derives its free carbon dioxide from various sources.

The interaction of the nitrogen and carbon sources, as they are added, leads according to equation I, to the formation of CO_2. By the process of nitrification itself, according to equation II, more free CO_2 is formed, while the atmospheric carbon dioxide

constitutes a source that for convenience shall be here named III. It is then, possible that, like all other organisms, the Nitrosococcus undergoes processes of anabolism and catabolism, in the latter probably giving off carbon dioxide by respiration: this source shall be here named IV.

1. $MgCO_3 + (NH_4)_2 SO_4 \rightleftarrows MgSO_4 + (NH_4)_2 CO_3 \therefore NH_3 + H_2CO_3 + H_2O + CO_2$

II. $MgCO_3 + 2HNO_3 \rightarrow Mg(NO_3)_2 + H_2CO_3 \therefore H_2O + CO_2$

Duclaux (1896) expressed the opinion, based upon the results of the work of Winogradsky and of Godlewsky, that if the organisms were not capable of utilizing the carbon of the carbonate this could be due to the fact that some difference might exist between the carbon dioxide of the atmosphere and that arising from equations I and II above. One point should nevertheless be mentioned as a criticism of this view i. e., that by allowing the carbon dioxide absorbant to be continually present in the system (as was done by Godlewsky and in the above experiments duplicating his work), the action of the bacteria will be dependent not on the quality of the gas but on the velocity of distillation of the latter from solution. With very large surfaces of the culture solutions and relatively large surfaces of the absorbent, the CO_2 tension in the gaseous phase of the system will be so slight that the organisms will not be in position to utilize the very slight traces temporarily to be found in the solution, before they are removed from contact with them.

The question therefore becomes a twofold one: the organism of nitrosofermentation apparently cannot develop in the absence of all traces of gaseous carbon dioxide, yet is able to develop in media free of all traces of fixed organic carbon as was shown by Winogradsky (unless the traces carried in the inoculum may be considered as sufficient to vitiate the results, a conclusion hardly justified in view of the care with which the author attempted to avoid this a priori objection).

But the question of the growth of the nitrosoferment in an environment only initially free of carbon dioxide is as yet an open one. In order to answer the theoretical question advanced by Duclaux, cultures in full nitrification are best adapted, since in these the CO_2-production from equations I and II is considerable.

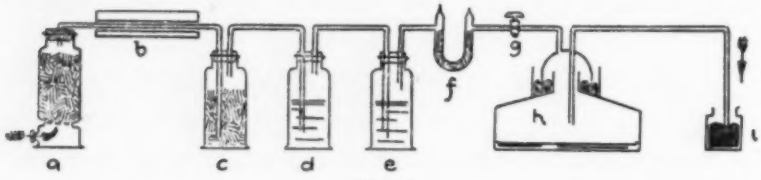

FIG. 1

a, Soda lime; *b*, furnace at 900–1000°C.; *c*, soda lime; *d–e*, concentrated KOH; *f*, soda lime; *g*, stop-cock; *h*, culture chamber; *i*, Hg. manometer.

Experiment 200

An apparatus prepared as is shown in figure 1.

The culture was in full nitrification when used and the experiment was started by aerating the flask for two and one-half hours, with air freed of CO_2 by passing through the train shown above. After aeration stopcock *g* was closed and the flask was incubated on a clinostat (Bonazzi, 1919 b). The quantities of nitrite formed before and after the treatment are given in table 3.

TABLE 3

	NITRITE NITROGEN FORMED IN 100 CC. OF SOLUTION DURING THE		
	Five days of preparation	Two days previous to treatment	Three days of treatment
Total	47.79	32.50	9.20
Formed per day	11.94	16.25	3.07

Experiment 229 and 229a

The apparatus used in these experiments were such that no negative pressures could obtain in the culture systems; the aeration train was similar to the one used in experiment 200,

with the omission of the furnace. The manometer of the previous experiment was also replaced by a paraffin-oil manometer guarded from the external carbon dioxide by a soda lime tube. A barium hydroxide bulb was placed between the culture flask and the suction pump to allow the detection of even small traces of CO_2. Aeration was carried out for two hours during which time the flasks were often shaken. After aeration, the bulb of $Ba(OH)_2$ was inserted and aeration continued for one half hour more during which time the hydroxide showed no turbidity. The results of these experiments are given in table 4 in which is also given the activity of the cultures in the period of preparation.

TABLE 4

	EXPERIMENT 229		EXPERIMENT 229 A	
	Nitrogen transformed to nitrite during			
	Two days previous to treatment	Seven days of treatment	Two days previous to treatment	Five days of treatment
	mgm.	*mgm.*	*mgm.*	*mgm.*
Total	8.44	18.66	11.33	19.72
Formed per day	4.22	2.67	5.56	3.94

It appears therefore that nitrification takes place normally at the expense of the carbon dioxide formed by the reactions in the culture medium and that therefore it must be assumed that the suggestion of Duclaux was based upon facts which could not well be utilized in the formulation of the hypothesis.

As may be seen, the cultures here used were in full and intensive nitrosofermentation and the carbon sources varied. Yet owing to the specific arrangement of the experiments, there should be a possibility of separating the supplies at their point of formation. In fact, if a very small inoculum were used in place of the very great ones used in the previous experiments, it should be possible to diminish appreciably if not to stop completely the formation of the supplies due to equation II. This was done in experiment 227–a.

Experiment 227–a

The apparatus used were made up according to figure 2.

Aeration was carried on for about one hour and the complete absence of CO_2 ascertained by means of the barium hydroxide bulb. The medium used was ignited and sterilized compost, moistened with sterile Omeliansky solution in the proportion of 40 cc. for each 100 gram of soil. One gram of basic $MgCO_3$ had been placed in each flask, while dry, before sterilization.

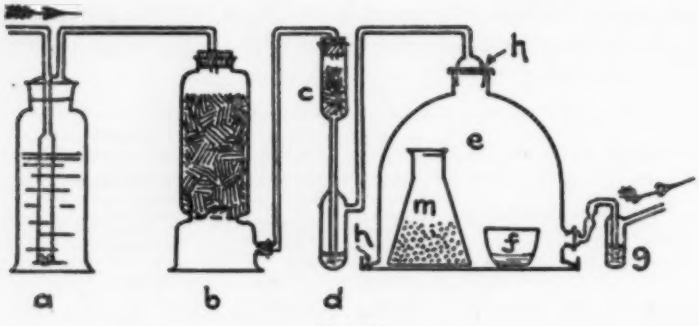

FIG. 2

a, Concentrated NaOH; *b*, soda lime; *c*, soda lime; *d*, paraffin oil seal; *e*, culture chamber with culture in —m; *f*, crucible with appropriate solution; *g*, $Ba(OH)_2$ guard for detecting CO_2; *h*, paraffin seals.

All flasks were inoculated with a soil suspension which contained negligible amounts of nitric or nitrous nitrogen. After inoculation, the cotton plugs were removed, the flasks placed under the bell jars, the seal made by pouring melted paraffin at the contact of jar and plate and aeration started. The arrangement of the flasks was the following:

No. 1. Check uninoculated.

No. 2. Air in system freed only of the initial CO_2.

No. 3. Air freed of CO_2 throughout, containing NaOH 50 per cent.

No. 4. Air containing only the CO_2 developing from the reaction of 5 mgm. Na_2CO_3 and an excess of H_2SO_4 taking place in *f*.

No. 5. Air with no change in composition (natural).

After thirty days incubation at 25°C. the nitrates were extracted, and determined by reduction and distillation. The results are given in table 5.

TABLE 5

NUMBER	TREATMENT	$NH_3 - N_2$ AT THE END	N_2 NITRIFIED
		mgm.	*mgm.*
1	Check	....	....
2	No CO_2 at start	8.77	1.28
3	No CO_2 throughout	9.75	0.44
4	CO_2 added	0.00	8.26
5	Natural air	0.07	14.57

System no. 4 requires a little discussion. Sulphuric acid was added in excess of the quantity needed for the neutralization of the Na_2CO_3, and apparently its action has been to absorb the ammonia which was distilling from the culture itself; the final nitrite content could be but low. It should also be stated that no attempt was made to recover all the residual ammonia in the soil. The quantity of nitrate formed in no. 3, if not accountable by an experimental error in analysis, would require, according to the ratio established by Winogradsky, a quantity of 0.01 mgm. of carbon as CO_2, a quantity which could easily have escaped immediate absorption by the alkali used.

The conclusions to be drawn from these experiments are, then: (a) that nitrification is completely checked by the complete absence of carbon dioxide in the system throughout the experimental period (when a concentrated alkali is present therein); (b) that when a small inoculum is used, in spite of the fact that ignited soil holds carbon dioxide by absorption, and when the supply of this gas is nil at the start, the phenomenon of nitrosofermentation is practically inhibited.

In direct consequence of these facts stands the conclusion that, since the inoculum was small and nitrification in no. 2 was negligible, contrary to the results of experiments 229 and 229a, and since in this case there was not an active production of carbon

dioxide by virtue of equation II, the latter source of the gas appears to be the one most active in furthering nitrification. This is probably attributable to the fact that it is directly connected with the life activities of the organism concerned.

The supply due to reaction I is, therefore, slowly utilized, probably because very rapidly distilled from the immediate surroundings of the cells; or a molecular rearrangement of the basic carbonate used may take place. These considerations would then place the process of nitrification dependent upon and secondary to the process of carbon assimilation. We shall have occasion to return to this point later in this paper.

According to Warington, nitrification of ammonia can take place only with ammonium carbonate, and the function of the carbonated base is to furnish that compound by reaction with other ammonium salts. Ashby found nitrification to take place in presence of ferric hydrate to a considerable extent, and also some nitrification of the ammonia absorbed by modeling clay alone (1907–1908).

Hopkins and Whiting (1916) also claim that nitrification will take place in presence of tri-calcium phosphate as a neutralizing substance. It is evident that in the experiments of the last named investigators ammonium carbonate could not be formed by reaction between the neutralizing base and the source of nitrogen, a fact that throws a doubt on Warington's assumption. The reaction leading to the formation of ammonium carbonate should then be directly connected with the nitrogen nutrition of the organism of nitrosofermentation rather than with the carbon nutrition. The question of the autotrophy and heterotrophy of the organisms is also intimately connected with these observations.

If it were possible to avoid, in a culture, reactions I and II, a step would be taken towards the understanding of the phenomenon that Winogradsky named, "chlorophyllic action without chlorophyll". The aim could be approached either by the use of a non-carbonated base or by allowing nitrification of the ammoniacal nitrogen in the form of hydroxide. Experiments in the hope that the second of these two assumptions might prove

practical failed to give satisfaction. In presence of $MgCO_3$. Mg $(OH)_2$ as a neutralizing base, ammonium hydroxide added in small amounts at a time was easily volatilized and removed from the nitrifying system: only 7 mgm. of nitrogen were transformed to nitrite in eleven days although 30 mgm. of nitrogen as ammonium hydroxide were added during this period, in small amounts ranging from 2 to 9.4 mgm. at one time. This source of ammonia even though undergoing slow nitrification did not seem to hinder the action of the organisms, since the same culture which had received the hydroxide additions was capable, after this treatment, of nitrifying in three days 2 cc. of a 10 per cent solution of ammonium sulphate for every 100 cc. of culture solution.

Although the results obtained pointed to the possibility of nitrification of ammonium hydroxide, it was thought that a study of a non-carbonated base to be used in the cultures would prove more satisfactory. Magnesium oxide was chosen since it has a lower solubility of the carbonate and would give results directly comparable to the conditions existing in the ordinary culture solution as used in the present paper. Calcium oxide and hydroxides as well as the oxides and hydroxides of the other metals were discarded *a priori* on account of their greater solubility and greater alkalinity. The aim was to use a base that would serve to neutralize the acids arising from the reactions taking place during nitrification rather than to exert its physiologically alkaline properties. That the oxide chosen is not toxic is to be assumed from the fact that the carbonate, used in all the previous experiments, contained one molecule of the oxide for every molecule of the carbonate.

Experiment 234

Six flasks with very large flat bottoms received 50 cc. of the ordinary Omeliansky solution made up with tap water. After sterilizing and cooling, flasks 1, 2 and 3 received the addition of a sterile suspension of Mg CO_3. Mg $(OH)_2$, while flasks 4, 5 and 6 received a sterile suspension of MgO. All except nos.

TABLE 6

NUMBER	TREATMENT		REACTION AFTER			
			Nine days		Thirteen days	
	Base	Inoculation	NH_3	N_2O_3	NH_3	N_2O_3
1	$MgCO_3$	Check uninoculated	**	0		
2	$MgCO_3$	Inoculated	0	**		
3	$MgCO_3$	Inoculated	0	**		
4	MgO	Check uninoculated	**	0	**	0
5	MgO	Inoculated	**	1	**	1
6	MgO	Inoculated	**	+	**	+

1 and 4 were inoculated with an active culture of the nitrite ferment and incubated at 25°C. Tests made at various intervals yielded the results given in table 6 where 0 signifies a negative result and 1, +, ** positive results of increasing intensity.

On a short period of incubation, although free atmospheric carbon dioxide was in contact with the solutions no appreciable nitrification took place, when magnesium oxide was used, while nitrification was active in the presence of the carbonate.

Experiment 240

The above cultures in which no appreciable nitrification took place (nos. 4, 5, 6) were each divided into two equal portions by means of sterile pipettes and placed, without reinoculation, in sterile flasks of equal diameter. One portion, left as control, received no addition whereas the other portions received various treatments, as is shown in table 7.

TABLE 7

NUMBER	TREATMENT	NITROGEN AS NITRITE AFTER SECOND PERIOD OF INCUBATION	FINAL AMMONIA REACTION
		mgm.	
4	Check uninoculated	0.77	**
5	$(NH_4)_2SO_4$ and MgO	6.22	?
51	$(NH_4)_2SO_4$ and MgO and $MgCO_3$	4.85	?
6	$(NH_4)_2SO_4$ and MgO and $(NH_4)_2CO_3$	2.50	+
61	$(NH_4)_2SO_4$ and MgO, $(NH_4)_2CO_3$ and $MgCO_3$	2.35	**

The results obtained in this, the second, period of incubation are striking. Ammonium sulphate will be nitrified, although slowly, in the presence of a non-carbonated base, the difference in the results of experiments 234 and 240 being due to the *time* factor. The lag in nitrite formation in cultures 51, 6 and 61 may be explained in either of two ways: (a) distillation of the ammonium carbonate, and (b) retarding effect of this compound with subsequent volatilization of considerable quantities of ammonia. In fact in the cultures 6 and 61 a concentration of ammonium carbonate was used such as to give 28 mgm. of nitrogen in 25 cc. of solution, a quantity twice as great as that of the ordinary Omeliansky solution.

The results relating to the nitrification in the presence of a non-carbonated base are then in accord with the findings of Ashby and of Hopkins and Whiting and it is possible that the atmospheric carbon dioxide, here, played an important rôle in the process of nitrification. That ammonium carbonate is nitrifiable as such should be assumed from the work of Warington, but additional proof is furnished by experiments here to be related.

Experiment 241

Fifty cubic centimeters of Omeliansky solution from which the ammonia source was omitted, were sterilized in 750 cc. Erlenmeyer flasks. After cooling they received sterile magnesium carbonate suspension or magnesium oxide and either 1 cc. of a sterile 10 per cent ammonium sulphate solution or 0.5 cc. of a 17.2 per cent ammonium carbonate[1] solution pasteurized at 60°C.

The arrangement of the experiments[2] and the results obtained are given in table 8.

[1] The carbonate used in all these experiments was a mixture of the normal carbonate and the carbamate of the following empirical formula: $(NH_4)_2CO_3$. $NH_4CO_2NH_2$.

[2] The term *capped* in this and other experiments refers to the mercury or paraffin oil seals used in order to avoid the escape of volatile substances from the nitrifying systems. Their efficiency may be noticed by a comparison of the results obtained in nos. 3 and 4 of table 8.

TABLE 8

NUMBER	TREATMENT	REACTION AFTER		NITRITE NITROGEN FOUND AFTER 31 DAYS
		10 days	19 days	
				mgm.
1	$(NH_4)_2SO_4$ and $MgCO_3$ open	1	±	12.25
2	$(NH_4)_2SO_4$ and $MgCO_3$ capped	0	0	4.44
3	$(NH_4)_2CO_3$ open	+	±	6.47
4	$(NH_4)_2CO_3$ capped	1	±	20.62
5	$(NH_4)_2CO_3$ and $MgCO_3$ capped	0	1	13.12
6	$(NH_4)_2CO_3$ and MgO capped	0	0	0.75
7	$(NH_4)_2CO_3$ and MgO capped	0	0	0.78

In no. 2 nitrification was, for some unknown reason, retarded, for, even after nineteen days no nitrite formation had taken place, so that the quantity found after thirty-one days was formed during the last twelve days of incubation. The action of the magnesium oxide on the nitrification of ammonium carbonate is a retarding one and this may be due to physical reasons. Distillation of the ammonia from the neutral or slightly acid solution takes place at a relatively fast rate, as is shown in no. 3 and in a solution made alkaline by the addition of MgO the distillation is too fast to allow any competition by the bacterial cells.

Since it is evident from these experiments that the ammonium carbonate can be utilized by the nitrosoferment even in the absence of a base, a study of the nitrification of this substance in the presence or absence of atmospheric carbon dioxide will lead to a better understanding of the function of the magnesium carbonate in the cultures undergoing nitrification. Besides, if ammonium carbonate were nitrified in the absence of atmospheric carbon dioxide it would be evident that this nitrogenous carbonated substance could furnish the carbon necessary for the life of the organism. The latter condition would also indicate the mode of action of the compound.

Experiment 244

A solution prepared as follows: sodium chloride 1 gram; dipotassium phosphate 0.5 gram; hydrated magnesium sulphate 0.51 gram; hydrated ferrous sulphate 0.364 gram; distilled water 500 cc. Fifty cubic centimeter portions pipetted into six large flat-bottomed Fernbach flasks fitted with paraffin oil seals.

After sterilization flasks 1, 2 and 3 received sterile $MgCO_3$. $Mg(OH)_2$ and 1 cc. of a sterile 10 per cent $(NH_4)_2SO_4$ solution, while nos. 4, 5 and 6 received only 1.5 cc. of a 6.75 per cent pasteurized solution of ammonium carbonate. Subsequently all were inoculated. Flasks 2 and 5 received a small container of concentrated KOH in the neck while flasks 3 and 6 were washed free of all carbon dioxide by aeration for one hour, the air issuing from the flasks at the end of this time causing no turbidity in $Ba(OH)_2$ when passed through it. Inoculation was made with an active culture of *Nitrosococcus*, and incubation was at 25°C. for fifteen days. Analysis of the cultures after this period gave the results presented in table 9.

TABLE 9

NUMBER	TREATMENT	NITRITE NITROGEN FOUND AT THE END OF INCUBATION PERIOD
	$MgCO_3$ and $(NH_4)_2SO_4$	
		mgm.
1	Normal air	16.98
2	CO_2 removed throughout experiment	0.41
3	Only the initial CO_2 removed	8.76
	$(NH_4)_2CO_3.NH_4CO_2NH_2$	
4	Normal air	7.17
5	CO_2 removed throughout experiment	0.47
6	Only initial CO_2 removed	8.59

Experiment 246

Erlenmeyer flasks of 750 cc. capacity were used with mercury seals, and 50 cc. of the solution used in the previous experiment. After sterilization, 1 cc. of the pasteurized solution of ammonium

carbonate used in experiment 244 was pipetted in each flask and, after inoculation, a container with concentrated KOH was placed in the necks of nos. 1 and 2. After incubation at 25°C. the solutions submitted to analysis gave the following results (table 10).

TABLE 10

NUMBER	TREATMENT	NITRITE NITROGEN FOUND AT THE END OF INCUBATION PERIOD
		mg/m.
1	All CO_2 removed throughout experiment	0.13
2	All CO_2 removed throughout experiment	0.38
3	Natural stagnant air	7.52
4	Natural stagnant air	6.24

Repetition of this experiment (experiment 249) yielded the results given in table 11.

TABLE 11

NUMBER	TREATMENT	NITRITE NITROGEN FOUND AT THE END OF INCUBATION PERIOD
		mgm.
1	All CO_2 removed throughout experiment	0.35
2	Natural stagnant air	16.63

DISCUSSION AND CONCLUSIONS

From the foregoing experiments the following considerations seem justified.

In the solution containing ammonium sulphate and magnesium carbonate removal of all traces of carbon dioxide causes nitrosofermentation to come to a standstill, whereas if only the carbon dioxide present at the start be removed but that developing through reactions I and II (given on page 483) be allowed to accumulate in the systems nitrosofermentation could continue in a ratio roughly proportional to the size of the inoculum. In fact, even with a very small inoculum some nitrification could be detected.

It should be assumed, therefore, that the carbon dioxide formed through these reactions can go to replace that removed at the start; the CO_2 tension in the liquid and gaseous phase soon approaching that state of equilibrium which is favorable to the action of the nitrite-forming bacteria. In the presence of KOH, or other CO_2 absorbent, these reactions are taking place, but the gaseous products formed in relatively small quantities are soon removed by the absorbent, the resulting CO_2 tension in the culture solution reaching the point where the normal activity of the organism is impossible. Under such conditions it is even to be doubted whether ammonium carbonate is formed as such and not immediately hydrolyzed and the products of hydrolysis distilled from reach of the cells before completion of the synthetic step in the reaction. This is evidenced by the fact that when Omeliansky solution containing ammonium carbonate as the source of nitrogen is exposed to a concentrated alkali in a closed system it rapidly changes from an acid reaction, to phenolphthalein, to a strongly alkaline reaction. If ammonium sulphate be the source of nitrogen and magnesium carbonate be added to the solution, the final result is the same, a very strong alkalinity being developed where at first only a weak one could be detected.

In the tables reported above it is evident that such conditions lead to a check on the process of nitrification. The carbonate-carbamate used in view of the hypothesis of Chodat under the above conditions of CO_2 removal did not lead to a nitrogen hunger. This is proved by the fact that a solution containing this compound, and incubated in a system in which KOH was present, contained after the incubation considerable quantities of ammonia as determined by the Nessler reagent, while a heavy precipitate was obtained by allowing a drop or two of the culture solution thus incubated to react with $Ba(OH)_2$. Some carbonate as such was therefore still in solution.

These last considerations lead to the conclusion that it is the carbon dioxide as such that is necessary to the organisms of nitrosofermentation and that it can be utilized only when the tension of this gas is above a minimum limit. Thus the nitrogen nutrition of the organism is closely related and in fact completely

dependent upon its carbon nutrition. The free carbon dioxide is not only necessary for growth but is also necessary for the performance of the normal oxidative functions peculiar to the cells. Therefore nitrosofermentation which is supposed to furnish the energy for the carbon assimilation can not even be established in the absence of free carbon dioxide. The small quantity of nitrite formation in the presence of KOH, if positive at all, must be considered as the result of an autooxidation of the cells themselves and a utilization of the energy thus liberated, for the process of nitrite formation, a process which soon comes to a standstill because of the strong negative pressure of the CO_2 outside the cell and of the subsequent diffusion of the intracellular material towards the outside, and also because of the strong alkalinity developed in the medium.

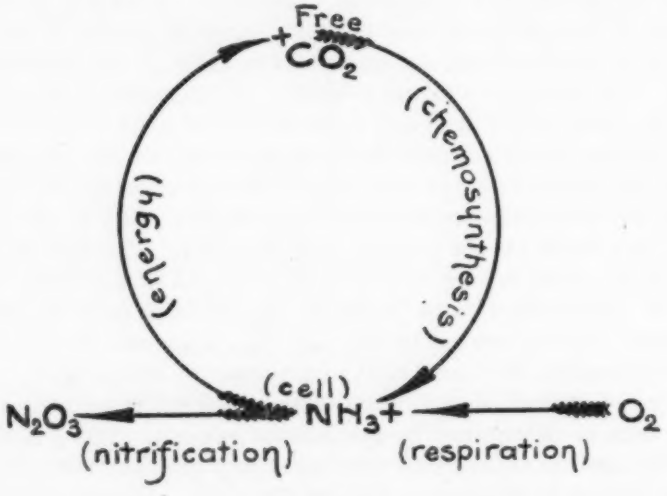

FIG. 3

This interpretation leads to a special conception of the life of the organisms of nitrosofermentation. When the optimum carbon dioxide tension is existent, the cells, during their life cycle, perform two synchronous functions; one of cellular respiration and one of carbon assimilation, the former serving for the initiation of the process of nitrosofermentation and subsequent

carbon assimilation by the second. Expressing these functions by means of a diagrammatic representation, figure 3 is obtained.

According to the above diagram, when the cell carbon is sufficiently large the process of chemosynthesis is endless, unless one of the end products is removed. Thus in the case of narcosis of the cell, respiration continues with degeneration and complete consumption of the available cell carbon, and when all such were consumed death of the cell would result. This condition of narcosis may be brought about by an excessive concentration of food substances or of cellular byproducts, with the cellular breakdown demonstrated by Bonazzi (1919-a) and by Gibbs (1919) and physiological inertia as has been shown by Boullanger and Massol (1903, 1904).

When the supply of free carbon dioxide is nil throughout the experiment the respiration process regulates chemosynthesis and the products of nitrosofermentation are in immediate relation to the quantity of cell substance respired and, since no carbon assimilation can follow, death of the cells results.

When ammonium carbonate is used as a source of carbon and the free carbon dioxide is continually removed by means of an absorbent there is a slow accumulation of the carbamate and fast removal of the ionized CO_2 (Macleod and Haskins 1906). Together with this there is a depletion of all the respirable stores in the cell, so that in the system the following substances will be found: $(NH_4)_2CO_3$, $NH_4CO_2NH_2$, cells, traces of respired C compounds, and NH_3. Since it has been experimentally proved that the cells cannot, under these conditions, assimilate this nitrogen (experiment 244, 246 and 249) the interpretation to be given to these facts is that the free carbon dioxide is closely tied up with the nitrogen nutrition of the organism and that in this condition it distills too fast for the cells to utilize it. It follows that if this distillation is prevented by mechanical means, there should then be possible some nitrification and chemosynthesis: this is what actually takes place in experiments fulfilling the required conditions.[3]

[3] Meyerhoff in Pflüger's Archiv f. Ges. Physiol. 1917. **166**, 240–280, found nitrification to proceed in the presence of 10 per cent NaOH solution, and the

The results obtained by the use of ammonium carbonate as a source of nitrogen and carbon, as well as those obtained with a non-carbonated base emphasize the fact that it is the free CO_2 that is utilized by the cells, a view substantially corroborated by the findings of Ashby and of Hopkins and Whiting reported above.

This free carbon dioxide is not necessary for the formation of ammonium carbonate, but for another purpose: chemosynthesis.

The oxidation of ammonia is to be considered as taking place in two steps: (a) one of respiration with resultant gain in energy and synchronous nitrogen absorption, (b) the other of nitrogen assimilation (nitrification proper) whereby oxidation of the absorbed nitrogen takes place, the utilized portion going to make up the following cell generations, nitrous acid is split off and excreted as a non-utilizable product, and energy is liberated. Therefore, if the free carbon dioxide were removed from a culture containing both ammonium carbonate, as a nitrogenous source, and large numbers of bacterial cells, respiration should be great enough to allow some nitrification to take place. If, on the other hand, the number of active cells were limited, nitrification would come to a standstill before a quantity of nitrites were formed detectable by the ordinary chemical means. Experimental evidence bears proof of the correctness of the above hypothesis.

SUMMARY

This paper reports a study of the functions of autotrophic carbon assimilation and nitrogen nutrition of the nitrosoferment. These functions are found to be intimately connected and mutually interdependent, the bacterial cell being unable to assimilate the abundant stores of nitrogen in a nutritive solution in the absence of "free" carbon dioxide, even though a carbonate as such, be present, in the medium. Consequently on the presence of this "free" carbon dioxide is dependent the process

present author found nitrification to proceed when the tube containing concentrated KOH was placed very near the mouth of the flask so that free circulation of the air was interfered with.

of nitrogen oxidation which follows the absorption and leads to the formation of nitrous acid and its salts.

REFERENCES

ASHBY 1907–1908 Jour. Agric. Sci. (Cambridge) **2**, 52–67.
BIEJERINCK AND VAN DELDEN 1903 Centr. f. Bakter., 2 Abt., **10**, 33–67.
BONAZZI 1919a Botan. Gazette, **68**, 194–207.
BONAZZI 1919b Jour. Bact., **4**, 43–59.
BOULLANGER AND MASSOL 1903 Annales d. l'Institut Pasteur, **17**, 492–515.
BOULLANGER AND MASSOL 1904 Annales d. l'Institut Pasteur, **18**, 181–196.
DUCLAUX 1896 Annales de l'Institut Pasteur, **10**, 414–416.
GIBBS 1919 Soil Science, **8**, 427–471.
GODLEWSKY 1892 Bull. Intern. Acad. Scie. Cracovie, 408–417.
GODLEWSKY 1895 Bull. Intern. Acad. Scie. Cracovie, 178–192.
HOPKINS AND WHITING 1916 Bull. 190, Illinois Agric. Exper. Stat., 395–406.
LOEW 1891 Botan. Central., **46**, 222–223.
MACLEOD AND HASKINS 1906 Jour. Biol. Chem., **1**, 319–334.
WINOGRADSKY 1890 Annales de l'Institut Pasteur, **4**, 257–275.
WINOGRADSKY AND OMELIANSKY 1899 Centr. f. Bakter., 2 Abt., **5**, 329, 377, and 429.

TOXINS OF BACT. DYSENTERIAE, GROUP III

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It was Shiga (1898) who first demonstrated the toxicity of cultures of the dysentery bacillus isolated by him in 1898. Later this toxin production has been studied by several investigators among whom are Neisser and Shiga (1903), Conradi (1903), Vaillard and Dopter (1903), Flexner and Sweet (1906) and Kraus and Doerr (1905). Recently Olitsky and Kligler (1920) have published a very interesting paper on this subject, showing that the dysentery bacillus of group I of Thjøtta's (1919) classification (the bacillus of Shiga) produces a soluble toxin (exotoxin) as well as an endotoxin, and that these two toxins act differently in rabbits. The former was shown to be a neurotoxin having no intestinal action, while the latter is an enterotoxin having no effect on the nervous system.

In the past the dysentery bacilli of group I were considered the only toxic forms of this bacillus, while those of the other groups (group II of Thjøtta's classification, i.e., the types of Flexner and Strong and the Hiss Y bacillus) were held to be atoxic.

In the following we will show that *Bact. dysenteriae* of group III as well as of group I produces toxins thus showing the relation of this group to the toxic strains of the *Bact. dysenteriae*.

Before going into the details of our experiments we will present the main characters of the bacillus of group III. It is, as in the case of other *Bact. dysenteriae* a Gram negative, nonmotile non-gas producing microbe, that forms acid in mannitol, maltose, glucose, and as a rule in sucrose. It does not produce indol and it grows in peculiar colonies having an irregular, crenated edge. It is toxic to a milder degree for rabbits and monkeys (Sonne).

This microorganism was evidently seen by Kruse in 1907 and called by him type E (Kruse, 1907); but it was first regarded as a definite type by Sonne in 1914 (Sonne, 1915) and classed by him in group III. Since then it has been described in France by d'Herelle (1916), in Norway by Thjøtta (1919) and in Sweden by Øhnell (1918).

EXPERIMENTAL

As a control on our technique, and as a confirmation of the results obtained by Olitsky and Kligler a strain of Shiga bacilli, the first of this form of dysentery bacilli to be isolated in Norway (by the authors) was examined.

The Shiga strain was grown in plain broth for eight days, then filtered, and the filtrate (Berkefeld) injected intravenously in the following rabbits with these results:

Experiment I. Exotoxin

RABBIT NUMBER	WEIGHT	AMOUNT INJECTED	FIRST DAY	SECOND DAY	THIRD DAY	FOURTH DAY
	grams	cc.				
1	3250	1.0	Very sick	Dead		
2	4000	0.5	Sick	Lies on side, does not move	Dead	
3	3600	0.25	Well	Quiet	Paresis of forelegs	Complete paralysis of anterior part of body
4	3250	0.125	Well	Well	Well	Well
5	3600	0.063	Well	Well	Well	Well

Thus it is proved that the filtrate acts as a neurotoxin, producing distinct paralysis; that a period of incubation precedes the development of these symptoms; and that the effect is dependent upon the amount of the filtrate employed. In none of these rabbits were intestinal lesions found.

The Shiga strain was grown on agar surface for twenty-four hours, washed off in saline solution, heated for 1 hour at 60°C., and finally

warmed at 37°C. for forty-eight hours. The suspensions were then filtered and injected into rabbits with the following results:

Endotoxin

RABBIT NUMBER	WEIGHT	AMOUNT INJECTED	FIRST DAY	SECOND DAY	THIRD DAY	FOURTH DAY
	grams	*cc.*				
1	2900	1.0	Dead			
2	3600	0.25—	Sick	Diarrhea	Very sick, diarrhea	Dead
3	3000	0.125—	Sick	Dead		
4	2800	0.063—	Sick	Diarrhea	Dead	

From these experiments it is to be noted that the prominent symptoms were intestinal in origin. At autopsy a marked hemorrhagic colitis was found, similar to that observed in dysentery in man in the stage prior to the development of necrosis. None of these rabbits showed nervous symptoms.

Thus the results were in accordance with the findings of Olitsky and Kligler, and we concluded that the conditions for obtaining a good yield of exotoxin and endotoxin were met by our technique.

We then proceeded with the study of the *Bact. dysenteriae*, group III. The reaction of the medium during the growth varied as follows:

TABLE 1

DAYS INCUBATION	pH
1	7.2*
2	7.3
3	7.5
4	8.0
5	8.0
6	8.0

*Medium before inoculation pH 7.6.

We thus find an initial acid production that is followed by a period of alkalinity. In the end the reaction is more alkaline than at the beginning of the experiment. This confirms the observations of Olitsky and Kligler who also found that the toxin production did not begin until the alkaline period had

set in. Following the technique of Olitsky and Kligler we collected our toxin after seven days' growth since too prolonged growth tends to yield mixed exotoxin and endotoxin. The broth was now filtered through a Berkefeld filter, the filtrate tested for sterility and the toxin thus prepared was injected into rabbits and white mice.

Experiment II. Injections into rabbits

RABBIT NUMBER	WEIGHT	AMOUNT OF EXOTOXIN INJECTED INTRAVENOUSLY
	grams	cc.
1	2500	3.0
2	2700	2.0
3	2600	1.0
4	1250	0.5

The results were as follows:

Four hours after the injection the animals became ill. All lay quiet without trying to move. Rabbits 1 and 2 also had quick labored respiration and appeared to be moribund. After this immediate and probably non-specific effect the animals returned to normal as to appearance. If they were disturbed, however, they did not jump about as normal rabbits do, but dragged themselves along with a distinct weakness of the hind legs.

The weakness of the hind legs was most distinct in the second and third day of the experiment; thereafter it gradually disappeared.

The loss of weight was as much as 500 grams (rabbit 1).

A control rabbit injected with 3 cc. of sterile broth did not show any symptoms.

Additional experiments were made but as the results were similar the protocols are not given. The injection of filtered broth cultures of dysentery bacilli of group III thus causes an effect in the rabbits characterized mainly by distinct paresis of the extremities. No diarrhea was observed and no deaths occurred.

Endotoxins of Bact. dysenteriae, group III. The endotoxin was prepared as follows:

Large flasks of agar (diameter 18 by 10.5 cm.) were incubated after inoculation with the strains used in the experiments on exotoxin. A

twenty-four hours' growth of the bacilli was emulsified in normal saline, and the emulsion placed in the incubator for autolysis for two days. It was then filtered through Berkefeld candles, tested for sterility and injected into rabbits.

Experiment III. Injections into rabbits

RABBIT NUMBER	WEIGHT	AMOUNT INJECTED	FIRST DAY	SECOND DAY	FOURTH DAY
	grams	*cc.*			
1	750	3	Dead		
2	700	2	Very ill, diarrhea	Improved	Well, weight 550 grams
3	800	1	Slight diarrhea	Well	Well, weight 850 grams
4	2850	3	Very ill, diarrhea	Improved	Well, weight 2650 grams
5	2250	2	Normal stool	Well	Well, weight 2100 grams

Thus, the rabbits after being injected with the endotoxins of the dysentery bacillus of group III showed intestinal disturbances as indicated by the diarrhea. One animal died following a large dose (3 cc.) after having shown a profuse blood-stained discharge from the intestines. One animal did not show any sign of diarrhea. In no case was there paresis, and recovery followed promptly.

Experiments on mice

Kraus and Doerr studied the effects of the toxins of the Shiga bacillus on hens and pigeons and found they were refractory. Doerr likewise found that guinea-pigs were not affected. We undertook the study of the effects of both the exo- and endotoxin on mice with the following results:

Experiment IV. Exotoxin

MOUSE NUMBER	AMOUNT OF EXOTOXIN INJECTED
	cc.
1	1.0
2	0.5
3	0.25
4	0.10
5	0.05
6	1.0*

* Sterile broth.

Four hours after the injection mice 1 to 5 had a profuse bloody and slimy discharge from the anus, the stool hanging in drops from the anal opening. All the mice were sick, huddling together and showing raised hair. Thirty-six hour after the injection the animals were all right. Mouse 6 did not show any symptoms at all.

Experiment V. Endotoxin

MOUSE NUMBER	AMOUNT OF ENDOTOXIN INJECTED	FIRST DAY	SECOND DAY	THIRD DAY
	cc.			
1	2.0	Dead		
2	1.0	Dead		
3	0.5	Very sick	Sick	Well
4	0.25	Very sick	Sick	Well
5	0.10	Very sick	Dead	
6	0.05	Sick	Sick	Well

All the sick mice had a diarrhea of blood-stained mucus after the injection, developing very soon (one and a half hours after injection). At autopsy there was enterocolitis and the lumina of the intestine contained blood-stained mucus.

Considering the rapid development of the symptoms in mice and the uniformity of the latter in both experiments it is probable that the symptoms were non-specific, at least where the exotoxin is concerned. As to the endotoxin, this certainly made the mice very sick and even killed them, the toxicity of the filtered broth thus being certain. However whether this toxic action was only due to the non-specific bacterial protein toxicity or indicated the specific action of a dysentery toxin we cannot state with certainty.

Antitoxins

Our next step was to study the production of antitoxins in the blood of immunized animals and to determine whether these antitoxins were capable of neutralizing the action of both the exotoxin and the endotoxin. For this purpose strong full-grown rabbits were used. The first rabbit receiving exotoxin died from a fulminating intoxication after three injections. The next animal bore the injections well and was given 10 doses of 1 cc.

each before the serum was drawn. An endotoxin animal also received 10 injections of 1 cc. each before the serum was used: and after these injections both these animals were apparently immune against the corresponding toxin.

Owing to a shortage of animals we have made only one experiment with rabbits. We chose to try endotoxin as this toxin showed more distinct symptoms of poisoning than the exotoxin.

ENDOTOXIN	ANTIENDOTOXIN	RESULTS
cc.	cc.	
3	1.0	Well, no diarrhea, loss of weight in 2 days, 50 grams.
3	0.1	Slight diarrhea, loss of weight in 2 days, 200 grams.

ENDOTOXIN	ANTIEXOTOXIN	RESULTS
cc.	cc.	
3	1.0	Very sick, diarrhea
3	0.5	Very sick, diarrhea.
3	0.1	Very sick, diarrhea, died in 26 hours after injection.
3	0	Very sick, diarrhea, lived

In this experiment the sick animals and the one death occurred in the tests made with heterologous toxin and antitoxin, while the animals that were injected with the mixture of homologous toxin and antitoxin were fairly well.

We are quite aware that our doses were large and our animals few. But we cannot free ourselves of the opinion that there was a distinct antitoxic action exercised by the sera from the animals immunized against the homologous toxins. The titration of this action in exact doses was hardly possible because of the mild action.

We now turned our attention to the effect of neutralizing sera on mice, which, as noted previously, acted in a non-specific manner to the action of exotoxin, and possibly in a specific manner to that of the endotoxin.

ENDOTOXIN	ANTIENDOTOXIN	RESULTS
cc.	cc.	
2	0.5	All animals well
2	0.1	All animals well
2	0.01	All animals well

ENDOTOXIN	ANTIEXOTOXIN	RESULTS
cc.	cc.	
2	0.5	Sick with diarrhea, lived
2	0.1	Sick with diarrhea, died within 24 hours

ENDOTOXIN	NORMAL SERUM	RESULTS
cc.	cc.	
2	0.5	Sick, diarrhea, lived

EXOTOXIN	ANTIEXOTOXIN	RESULTS
cc.	cc.	
2	1.0	Slight diarrhea, lived
2	0.5	Well
2	0.1	Died in 24 hours
2	0.05	Well

EXOTOXIN	ANTIENDOTOXIN	RESULTS
cc.	cc.	
2	1.0	Died in 24 hours
2	0.5	Sick without diarrhea
2	0.1	Sick without diarrhea
2	0.05	Sick without diarrhea
2	0.005	Died in 24 hours

The experiment seems to show a distinct protective action of the antiendotoxin serum against its homologous toxin, while the control tests with endotoxin and antiexotoxin serum did not show protection. It seems therefore justifiable to consider the toxic effect of the extract of the dysentery bacilli (the "endotoxin") as a specific action due to the endotoxins of the dysentery bacillus rather than to a non-specific protein toxicity.

In the tests with exotoxin we did not find any distinct neutralization of the toxic effect of the broth injected. This fact might be due to the short period of immunization of our serum

yielding animals. In comparison however with the non-specific appearance of the symptoms of the exotoxin injection we find that this lack of neutralizing effect of the antiexotoxin serum makes it still more probable that the reaction of the mice after injection of exotoxin must be characterized as a non-specific reaction.

CONCLUSIONS

1. *Bact. dysenteriae* of group III produces both exotoxin and endotoxin.

2. The endotoxin is the most marked in effect and produces intestinal symptoms in rabbits and mice, while the exotoxin of this group is milder in action, producing pareses in rabbits, while mice react non-specifically to it.

3. The repeated injections of these toxins over a relatively short period of time render rabbits immune. The sera of these animals show a weak protective action against the homologous toxins.

REFERENCES

CONRADI, H. 1903 Deut. med. Woch., **29**, 26.
D'HERELLE, H. 1916 Ann de l'Inst. Pasteur, **30**, 145
FLEXNER, S., AND SWEET, J. E. 1906 Jour. Exp. Med., **8**, 514.
KRAUS, R., AND DOERR, R. 1905 Weiner Klin. Woch., **18**, 514.
KRUSE, 1907 Deut. Med. Woch., **33**, Nos. 8 and 9.
NEISSER M., AND SHIGA, K. 1903 Deut. med. Woch., **29**, 61.
ØHNELL, H. 1918 Kliniska och bakteriologiska bidrag till kännedomen om dysenterien i Sverige, Stockholm.
OLITSKY, PETER K., AND KLIGLER, I. J. 1920 Jour. Exp. Med., **31**, 19.
SHIGA, K. 1898 Centr. f. Bakt., **23**, 599.
SONNE, C. 1915 Centralbl. f. Bakt., **75**, 408.
THJØTTA, TH. 1919 Jour. Bact., **4**, 355.
VAILLARD, L., AND DOPTER, C. 1903 Ann. de l'Inst. Pasteur, **17**, 486.